THE UNIVERSITY OF ALBERTA

ANALYSES OF LYMPHOCYTE PROLIFERATION

by

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To my parents, Ida and Harold Gerhart, who are such fine examples to follow, and to my loving wife Agnes, who shares all.

ABSTRACT

Control of the mammalian immune system is accomplished, to a large extent, by control of immunocyte proliferation. The stimulation of murine thymocytes by Concanavalin A (Con A) is a simple model of immune system proliferation and thus is a good model for the study of proliferation control. Before proliferation control can be studied, the kinetic parameters of proliferation of the Con A stimulated thymocyte population must be established. The absolute rate of thymocyte DNA synthesis as a result of Con A stimulation was measured as picomoles of thymidine incorporated per hour per 10^6 cells. The time course of thymocyte stimulation by optimal concentrations of Con A was established. Variations in the time course of stimulation as a result of variation in Con A or thymocyte concentration were studied.

It was important to establish whether the thymocyte DNA synthesis induced by Con A involved the passage of thymocytes through more than one S-phase. A technique of cell cycle analysis involving incorporation of ^{3}H-thymidine and 5-bromo-2'-deoxyuridine (BrUdR) into thymocyte DNA was developed. The controls for this technique indicated that thymocyte metabolism was not affected by incorporation of these thymidine analogues. The technique established that thymocytes do pass through at least two S-phases during Con A stimulation, with a growth fraction of 0.67. The mean cell cycling time was found to be 12.5 hours. Heterogeneity of thymocyte cycling time was observed. This suggested that thymocyte cycling time is not inherited but is based on a random selection of a limited range of

cycling times. Cell cycle analysis also established that thymocytes, once stimulated by Con A, are capable of initiating a response and continuing to cycle after Con A has been removed from culture.

The BrUdR technique of cell cycle analysis was applied to the cycling time analysis of sheep red blood cell (SRBC)-stimulated murine spleen cell cultures. The cell cycling time of such cultures was found to be 13 hours. Attempts were made to establish the cycling time of the SRBC-specific spleen cell subpopulation but the techniques of cell selection necessary for such an analysis proved inadequate.

A murine myeloma cell line was also studied with the BrUdR technique. The myeloma cells exhibited a cycling time of 14.3 hours and a growth fraction of approximately 1.00. This was in agreement with the doubling time established by microscopic cell counting.

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LIST OF ABBREVIATIONS AND GLOSSARY

A cell	accessory cell or adherent cell (macrophage)
antibody	a protein which can be incited in an immune system by an antigen and which reacts specifically with that antigen
antigen	any entity which can incite an immune system to produce specific antibodies and which reacts with those antibodies
B cell	bone marrow-derived or " bursa-equivalent " lymphocyte
BrUdR	5-bromo-2'-deoxyuridine
cAMP	adenosine-3',5'-cyclic monophosphate
CBA/J	inbred mouse strain
CdR	1-β-2'-deoxy-D-ribofuranosylcytosine
Ci	curie: 2.22×10^{12} disintegrations per minute
complement	serum proteins which, when used in collaboration with antibodies specific for a foreign cell, cause the cytolysis of that cell
Con A	Concanavalin A, a mitogen for thymus-derived lymphocytes
dbcAMP	N^6-O^2'-dibutyryl-adenosine-3',5'-cyclic monophosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ficoll	a high molecular weight (∿ 400,000) sucrose polymer
g	gravity units
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H/H	DNA possessing the density label BrUdR in both strands
H/L	DNA possessing the density label BrUdR in one strand
L/L	native density DNA
Lymphoprep	a commercial preparation of ficoll and hypaque (sodium diatrizoate)

M-D	Mishell - Dutton balanced salt solution
αMG	α-methylglucopyranoside
mitogen	any agent which incites a cell to enter S-phase
M-phase	the time of the cell cycle involved with mitosis
plaque	a clear area in an SRBC lawn resulting from a central cell secreting antibody directed against SRBC
pmole	picomole
pfc	plaque-forming cell
PHA	Phytohemagglutinin, a mitogen for thymus-derived lymphocytes
RNA	ribonucleic acid
rosette	a lymphocyte coated with SRBC as a result of SRBC-specific antibodies at the lymphocyte surface
sCon A	succinylated Concanavalin A
S-phase	the time of the cell cycle involved with the replication of nuclear DNA
SRBC	sheep red blood cells
T7	a DNA-containing bacteriophage
T-cell	thymus-derived lymphocyte
TdR	1-β-2'-deoxy-D-ribofuranosylthymine
Thy-1	thymus-derived lymphocyte surface antigen
Thy-1 serum	a serum which contains antibodies specific for the Thy-1 cell surface antigen
Tris	tris (hydroxymethyl) aminomethane
UR	1-β-D-ribofuranosyluracil
Zap Isoton	a commercial detergent preparation suitable for selectively lysing red blood cells

SOURCES OF MATERIALS AND REAGENTS

Sources	Materials
Aldrich Chemical Co.	8-Hydroxyquinoline
J. T. Baker Chemical Co.	General chemicals, reagents and buffers
Becton Dickinson and Co.	16 x 100 mm glass culture tubes
Calbiochem	α-Methylglucopyranoside
Coulter Electronics of Canada Ltd	Zap Isoton
Eastman Kodak	Succinic Anhydride
Fischer Scientific	Trichloroacetic Acid
Grand Island Biological Co.	Culture medium ingredients, penicillin-streptomycin and guinea pig complement
L'industrie Biologique Francaise	Agarose
Lux Scientific	Plastic T flasks and petri dishes
Miles-Yeda	Concanavalin A
New England Nuclear	Radiochemicals and Omnifluor
Nyegaard and Co.	Lymphoprep
Pharmacia Fine Chemicals	Ficoll 400
Pierce	Cesium Chloride
P. L. Biochemicals	5-Bromo-2'-deoxyuridine and 2'-deoxycytosine
Raylo Chemicals	2'-Deoxythymidine and Uridine
Schering	Gentamycin
Schwartz / Mann	N^6-$O^{2'}$-dibutyryl-adenosine-3',5'-cyclic monophosphate
Sigma Chemical Co.	Hepes
Standard Chemical Co.	Ethanol

Sources	Materials
Whatman	Glass fiber and paper filter discs
Worthington Biochemicals	Pancreatic DNAase
A generous gift of Ms. Jennifer Shaw, Dept. of Immunology, U. of Alberta	Thy-1 serum
Health Sciences Animal Center, University of Alberta (purchased from the Jackson Laboratory, Bar Harbor, Maine)	CBA/J mice
A generous gift of the Dept. of Immunology, U. of Alberta	Sheep Red Blood Cells and Carbonyl Iron

CHAPTER I

INTRODUCTION

I.1 The Study of Cell Proliferation

I.1.1 General Cell proliferation is involved, at some stage, in the developement of most differentiated cell systems. For this reason, the study of such systems must include a thorough understanding of the factors involved in the initiation, maintenance and termination of cell proliferation.

The techniques currently employed in the study of cell proliferation are limited in many respects. The simplest method of estimating cell proliferation is direct cell counting. This method is useful, however, only when studying entire cell populations or subpopulations of cells which exhibit distinguishing characteristics. Also, studies of cell proliferation based on direct cell counts may be compromised by the expansion of the cell population through differentiative activity rather than proliferative activity.

DNA synthetic activity has been employed as a measure of cell proliferation. In complex cell populations, however, the estimation of cell proliferation by monitoring DNA synthetic activity may be compromised by the contribution of cells other than those of interest. Also, the fraction of the absolute DNA synthetic rate which can be obtained through incorporation of exogenous deoxyribonucleosides must be established before cell proliferation and DNA synthetic activity may be equated.

The most common technique for the study of the kinetics of cell proliferation involves autoradiographic enumeration of metaphase (M-phase) arrested cells at time intervals after incorporation of radiolabeled deoxyribonucleoside (Quastler & Sherman, 1959; Borum, 1973; Bryant, 1971). This technique will yield a large amount of cell cycle kinetic data, but it does so under the influence of chemical manipulation of the cell's proliferative mechanism. Often the cycling of in vitro cell populations is synchronized at the outset of the experiment by release from S-phase block (Gentry et al., 1965; Till et al., 1963; Sinclair, 1967). The method of analysis requires that the cell population be arrested in M-phase at the end of the experiment, usually by the action of colcemid (Taylor, 1965). Such chemical manipulations may influence the proliferative mechanism such that the kinetic data obtained may not be representative of the freely cycling cell population (Barr, 1968; Tobey et al., 1967; Kato & Yosida, 1970).

I.1.2 Rationale for this work This thesis defines a model cell system suitable for the study of cell cycle kinetics. A technique is then described which can be applied to asynchronous cell populations to reveal cell cycle kinetic data, at the DNA level, without chemical disruption of the proliferation mechanism. The limitations of this technique are explored through its application to other cell systems.

I.2 The Choice of a Model Population

I.2.1 The immune system The purpose of an immune system is to inactivate any foreign antigen which enters the system. Antigen inactivation is often accomplished through a humoral immune response.

This involves the production of antibody which reacts specifically with the foreign antigen.

The immune system was chosen for study in this thesis because it possesses several interesting properties. Firstly, it is capable of responding to antigen only upon exposure to that antigen (Britton et al., 1973). Antigen stimulates differentiation and proliferation of immunocytes to a state capable of prompt antigen neutralization. Elimination of the antigen results in a return of the immune system to a less active state which is capable of immediate secondary response should the antigen be re-introduced.

The second interesting property of the immune response is its specificity. Regardless of the intensity of the response to a specific antigen, the majority of the immune system appears unaffected. Immune specificity is determined by cellular selection on the basis of cell surface antigen receptors (Nussenzweig, 1974; Warner, 1974). In this way, the differentiative and proliferative stimulation resulting from exposure to antigen is limited to the receptive immunocyte subpopulations.

The third area of interest in the immune response explores the fact that a variety of cell types act in concert in the response to an antigen. Bone marrow-derived cells (B-cells) give rise to antibody-producing cells (Nossal et al., 1968). Thymus-derived cells (T-cells) (Schrader, 1975; Katz & Benacerraf, 1972) and adherent cells (A-cells or macrophage) (Miller & Mitchell, 1968) are involved in the response. This synergy, which involves such concepts as antigen processing, antigen presentation and antigen recognition, is necessary for the generation of an effector cell population capable

of efficient antigen elimination.

The mounting of an efficient immune response involves a rapid proliferation of only the clone of immunocytes which exhibit specificity for the antigen. Therefore, a study of the control of proliferation in the immune response is a reasonable point from which to begin to understand the immune response as a whole. Since the immune response is a very complex phenomenon, a study of the biochemical basis of the control of proliferation demands either analysis of the individual components of the system or investigation of a simpler representative model.

I.2.2 The murine thymocyte population Mitogen stimulation of lymphoid cells appears to be a valid model system of the immune response. The three basic areas of interest in the immune response are adequately depicted by the mitogen-lymphoid cell model. First, the time course of the response of lymphoid cells to mitogen parallels the initial lag period, the period of proliferation of the responsive cells and the return to basal levels of activity exhibited by the response of the immune system to antigen (Lindahl-Kiessling, 1972). Secondly, the response of lymphoid cells to different mitogens exhibits some of the cellular specificity found in the immune response to antigen (Lindahl-Kiessling, 1972; Jacobsson & Blomgren, 1972; Jacobsson & Blomgren, 1974). Thirdly, as in the immune response, there is evidence for multicellular processing of the mitogenic signal resulting in the stimulation of one specific subpopulation of cells (Mills et al., 1976).

The lymphoid cell system chosen for the model was the murine thymocyte system. The choice was not based on the extent of mitogen

responsiveness since both spleen and lymph node cells have been found more responsive to mitogens than thymocytes (Jacobsson & Blomgren, 1974; Weksler et al., 1974). Rather the choice was based on the fact that the thymus contains few B-cells and A-cells (Cantor & Boyse, 1975). This reduces the cellular complexity of the model system. Also, thymocytes are relatively isolated from the efferent aspects of the in vivo immune system (Raviola & Karnovsky, 1972). This isolation eliminates variabilities in the in vitro responsiveness of the thymocytes to mitogen which might exist should the thymocytes be subject to in vivo stimulation by circulating antigen.

The mammalian thymus is the site of proliferation and differentiation of multipotential bone marrow stem cells into distinctive thymus-derived cells (T-cells) (Wu et al., 1968). The thymus has been shown to be both necessary (Wortis et al., 1971) and sufficient (Ritter, 1971) for the production of functional T-cells. On the basis of structure and cell population, four distinct regions of the thymus are distinguishable (Clark Jr., 1973). The subcapsular cortex is the chief site of thymopoiesis and is composed of quickly proliferating large lymphocytes. The inner cortex is crowded with small nonproliferating lymphocytes interspersed with large mitotic cells. The large degree of cell death in this region is associated primarily with adrenal cortical hormone secretion. The medulla is the region where thymocytes lose some thymus-specific cell surface antigens and gain the general cell surface murine histocompatability antigens. The perivascular connective tissue is the site of emigration of thymocytes into circulating blood. Proliferation due to circulating antigen is evident in this region.

The cortisone insensitivity of both mitogen responsive cells and only the medullary subpopulation of thymocytes (Jacobsson & Blomgren, 1972) is an indication that the medullary thymocytes are the most likely contributors to the mitogen response.

I.2.3 The thymic mitogen Concanavalin A The plant lectin Concanavalin A (Con A) was chosen as the mitogen for this model system because of the knowledge already available concerning its structure and function. Con A is most readily isolated from the Jack Bean (Agrawal & Goldstein, 1965). The amino acid sequence and X-ray crystallographic structure (Edelman et al., 1972; Cunningham et al., 1975) indicate that Con A exists as a tetramer above pH 7. Each monomer (molecular weight 25,500) possesses calcium, manganese and carbohydrate binding sites.

The in vitro response of thymocytes to plant lectins such as Phytohemagglutinin (PHA) (Yutoku et al., 1974; Andersson et al., 1972) and Con A (Juhlin, 1976; Gunther et al., 1974) has been compared (Lindahl-Kiessling, 1972; Jacobsson & Blomgren, 1974). Con A does not enter cells to exert its mitogenic effect (Greaves & Bauminger, 1972). At optimal stimulatory concentrations, 10^5 Con A tetramers are bound to each lymphocyte (Betel & Van Der Berg, 1972) while 10^6 - 10^7 tetramers can be bound per cell under saturating Con A concentrations. The responsibility for the binding of Con A to thymocyte membranes has been assigned to a cell surface glycoprotein of 55,000 molecular weight (Schmidt-Ullrich & Wallach, 1976).

The mechanism of Con A stimulation is still under study (Edelman et al., 1973). However, some of the early events which have been

associated with the stimulatory process are well defined. These events include alteration of membrane associated activities (Wettenhall & Slobbe, 1976; Painter et al., 1976), increases in membrane transport, increases in phospholipid syntheses and increased calcium influx (Hadden et al., 1974).

It has been established with circulating lymphocyes (Powell & Leon, 1970; Lindahl-Kiessling, 1972) and rat lymph node cells (Novogrodsky & Katchalski, 1971) that Con A must be in contact with the responding cell for at least 20 hours to exert maximal stimulation of DNA synthesis after 2 - 3 days of culture. Some stimulation of DNA synthesis is observed even if the responding cells are exposed to Con A for only 10 - 20 hours. This indicates the existence of an irreversible mitogenic commitment induced within a responding cell system by Con A.

I.3 The Application of 5-Bromo-2'-deoxyuridine to the Study of Cell Cycling Analysis of 5-bromo-2'-deoxyuridine (BrUdR)-containing DNA is a valuable tool for the study of DNA synthetic patterns. Incorporation of BrUdR can be studied through the buoyant density change of the DNA (Rownd, 1967) or through the reduction in fluorescence of DNA-associated dyes (Craig-Holmes & Shaw, 1976). The incorporation of BrUdR has been used to study the conservation of the order of replication of DNA in human transformed cells (Mueller & Kajiwara, 1966), slime mold (Braun & Wili, 1969) and Escherichia coli (Nagata & Meselson, 1968) and in the study of the restimulation of lymphocytes (Munakata & Strauss, 1972). The results indicate that analysis of BrUdR-containing DNA may facilitate cell

cycle analysis of asynchronous cell populations. The study of asynchronous cell populations eliminates the need for the chemical manipulations (cell synchronization and M-phase arrest) which may compromise cell cycling data.

The presence of BrUdR in tissue culture has, in some situations, caused specific cellular abberations. There is evidence that incorporation of BrUdR into DNA can increase the affinity of DNA for DNA-binding proteins (Lin & Riggs, 1972; David et al., 1974). This is a reasonable model to explain some of the inhibition of differentiative function which BrUdR can induce (Clark & Nudrud, 1974). The presence of BrUdR has been shown to induce differentiation of the neuroblastoma C 1300 in the absence of DNA synthesis (Schubert & Jacob, 1970). It is suggested that this may result from a BrUdR-induced alteration in the carbohydrate metabolism of the neuroblastoma cell surface proteins.

With these possible BrUdR side effects in mind, the application of analysis of BrUdR-containing DNA to the study of cell cycle kinetics was investigated.

CHAPTER II

THE NUCLEIC ACID SYNTHETIC ACTIVITY OF CONCANAVALIN A STIMULATED MURINE THYMOCYTES

II.1 Introduction

This thesis is concerned with the study of cell proliferation. The Con A stimulated thymocyte model system was chosen for the study of cell proliferation because of its close relation to the immune response and because of the knowledge already available concerning thymocytes and Con A. Chapter II describes investigations of the rate of DNA synthesis resulting from Con A stimulation of thymocytes. The validity of monitoring DNA synthetic activity by means of incorporation of exogenous thymidine into acid insoluble thymocyte material was explored. The rate of incorporation of thymidine at saturating concentrations was measured chemically.

Having established a technique for assaying DNA synthetic activity in this system, the parameters affecting DNA synthetic activity were studied. This included exploration of the effects of 2-mercaptoethanol, which has been shown to enhance *in vitro* immune responses (Click et al., 1972) and variation of mitogen concentration on the DNA synthetic activity of Con A stimulated thymocytes. The time course of the response of thymocytes to Con A was studied in terms of the effects of Con A concentration and the effects of the succinylated derivative of Con A (sCon A). This Con A derivative is less toxic than Con A and therefore permits saturation of the Con A mitogenic signal (Gunther et al., 1973).

The co-operation of thymocytes in the processing of the stimulatory Con A signal was investigated by varying the density of cells on the culture surface and monitoring the effect on DNA synthetic activity.

II.2 Methods

II.2.1 Radionucleoside preparation ^{3}H-Thymidine, ^{3}H-5-bromo-2'-deoxyuridine, ^{14}C-thymidine and ^{3}H-uridine were combined with the appropriate nucleoside to obtain solutions with specific activities of 20 - 50 cpm / pmole.

II.2.2 Culture labeling and harvest Thymocyte cultures were labeled by adding the appropriate radioactive nucleoside to the concentration indicated. After 2 - 4 hours of incorporation, the cultures were chilled to 4°C to stop further nucleic acid synthesis. Individual cultures were diluted 2 fold with 0.15 M sodium chloride and filtered through 2.5 cm glass fiber filters. The filters were washed twice with 5 ml of 0.15 M sodium chloride, thrice with 5 ml of 5% trichloroacetic acid, twice with 2 ml of 95% ethanol and dried.

II.2.3 Quantification of incorporated radionucleosides The acid insoluble material retained on glass fiber or paper filters was monitored for radioactivity in 5 ml of scintillation fluid (14.4 g of Omnifluor / 3 L of toluene). The scintillation of each sample was monitored for 5 minutes in a Beckman LS-250 scintillation counter. The conditions used yielded a counting error of less than 2% at the 99% confidence level for stimulated cultures. The resulting radioactive cpm data were corrected for isotope overlap when multiple radionucleosides were used and converted into the normalized units of

" picomoles of nucleoside incorporated per hour of labeling per million cells originally cultured " (pmole / hour / 10^6 cells). The rate of incorporation of exogenous thymidine can vary considerably in cultured thymocytes due to in vivo variations in metabolic and hormonal activity proir to culturing. Normalization of radionucleoside incorporation data to pmole / hour / 10^6 cells exposes such variability and allows an estimation of the absolute DNA synthetic rate of the tissue. The average standard deviation of triplicate samples in experiments examining DNA synthetic activity was 13%. Scintillation analysis of a factory tritium toluene standard established the counting efficiency to be 45% in the open channel.

II.2.4 Tissue culture conditions

II.2.4.1 Culture medium Thymocytes were cultured in Eagles Minimal Essential Medium containing Earle's Balanced Salt Solution. In addition, the medium contained 36 mM sodium bicarbonate, 10 mM Hepes buffer (pH 7.3), 0.43 mM sodium pyruvate, 10 units / ml penicillin, 10 µg / ml streptomyocin, 40 µg / ml gentamycin sulphate and 8.5% heat inactivated (56 °C for 30 minutes) fetal calf serum. Routinely, 2-mercaptoethanol was added to a final concentration of 10 µM.

II.2.4.2 Thymocyte isolation and culturing Thymuses were removed from 6 - 8 week male CBA/J mice which had been sacrificed by cervical dislocation. Thymuses were dispersed in 12 ml of culture medium by passage of diced tissue through fine stainless steel mesh. The dispersed tissue was allowed to settle for 10 minutes in a 15 x 75 mm plastic tube. During this time, connective tissue and clumped cells settled out from the cell suspension. The top 11.5 ml was removed and the cells were collected by centrifugation at 90 g for 7

minutes. The cell pellet was suspended in 10 ml of fresh medium. After settling for 10 minutes, the top 9.5 ml was removed and the cells were counted under methylene blue-acetic acid nuclear staining on a Spencer Haemocytometer. Viability tests were performed using eosin Y (0.15%) exclusion (Hanks & Wallace, 1958).

All cultures were in 16 x 100 mm round bottomed disposable glass tubes fitted with loose metal closures. Routine cultures consisted of 2 ml of medium containing 1 x 10^6 nucleated cells / ml. All culturing was at 37 °C in 100% relative humidity and an atmosphere of 10% carbon dioxide in air. Alternate culture methods employed flat bottomed glass vials (surface area 2.27 cm^2) or the Diener-Marbrook system. In the Diener-Marbrook system, cells are placed on a dialysis membrane suspended in a large volume of culture medium (Marbrook, 1967; Diener & Armstrong, 1969).

II.2.4.3 Mitogens

II.2.4.3.1 Concanavalin A Purification of commercially obtained Con A was not attempted but activation of Con A (Uchida & Matsumoto, 1972) was performed as follows: Con A was dissolved in 25 mM Hepes (pH 7.3) and dialysed for 40 hours at 4 °C against several liters of 25 mM Hepes containing 10 mM manganese chloride and 20 mM calcium chloride. After dialysis, excess salts were removed by vacuum dialysis of the Con A solution against 25 mM Hepes.

II.2.4.3.2 Succinyl Concanavalin A The succinylation of Con A (Gunther et al., 1973) was performed as follows: Con A (100 mg) was dissolved in 20 ml of saturated sodium acetate. The insoluble material was removed by centrifugation. The supernatant

plus 5 ml of saturated sodium acetate was added to 30.5 mg of succinic anhydride and the mixture was stirred for 2 hours at room temperature. The mixture was dialysed for 20 hours against water, then lyophilized. The resulting white, flocculent material was dissolved in 20 ml of saturated sodium acetate and the insoluble material was removed by centrifugation. The supernatant was added to 30 mg of succinic anhydride and stirred at room temperature for 1.5 hours. The mixture was dialysed for 20 hours against water, then vacuum dialysed for 12 hours against 25 mM Hepes (pH 7.3).

The concentration of mitogen solutions was determined spectrophotometrically assuming a 1% extinction coefficient (280 nm) of 12.4 for both Con A and sCon A (Yariv et al., 1968). Con A and sCon A were stored in the lyophilized state at -20 °C.

II.2.4.3.3 Sedimentation velocity analysis

Sedimentation velocity analysis of Con A and sCon A was performed in a Beckman model E analytical ultracentrifuge. Samples were analysed concurrently using a 2 place titanium rotor (AN-H) equiped with a 1° positive wedge window. The analysis was performed at 60,000 rpm for 80 minutes in a 25 mM Hepes buffer (pH 7.3) at 20 °C.

Con A (1.02 mg / ml) revealed a major component of 6.37 S and a minor component of 4.10 S. Succinyl Con A revealed a single component of 3.27 S when analysed at 4.0 mg / ml. These results agree reasonably well with the literature values [Con A (pH 5.6) - 3.9 S, Con A (pH 7.4) - 6.1 S and sCon A (pH 7.4) - 4.0 S (Gunther et al., 1973)]. The low molecular weight (4.1 S) contaminant in the Con A sample indicates the existence of Con A

fragments. The lower than expected sedimentation coefficient obtained from the analysis of the sCon A sample may be a result of the low ionic strength of the analysis buffer or concentration nonideality.

II.3 Results

II.3.1 Conditions of thymidine incorporation

II.3.1.1 Thymidine saturation Thymocyte cultures were brought to 0.5 - 20 μM ^{3}H-TdR at 47 hours. After 3 hours of labeling, the cultures were harvested. The double reciprocal plot of figure 1 describes the relation between exogenous thymidine concentration and incorporation of thymidine into acid insoluble material. Thymidine incorporation follows simple saturation kinetics. The thymidine concentration yielding half maximal incorporation rate is approximately 0.6 μM. A thymidine concentration of 10 μM, which yields 90% of the maximal incorporation rate, was chosen for use in pulse labeling experiments. It was assumed that exogenous thymidine underwent metabolism involved only with DNA synthesis therefore all acid insoluble tritium must be in DNA.

II.3.1.2 Thymidine pulse label duration At 47 hours, triplicate thymocyte cultures were exposed to 3.4 μM ^{14}C-TdR for 15, 60 or 120 minutes. The rate of thymidine incorporation has reached the steady state value during 15 minutes of labeling (table 1).

The comparison of long and short term ^{3}H-TdR incorporation was performed as follows: Triplicate 2 - 3 hour pulse labelings were performed at 40, 44, 48 and 52 hours employing 10 μM ^{3}H-TdR. On the basis of the incorporation rates at these times, the predicted

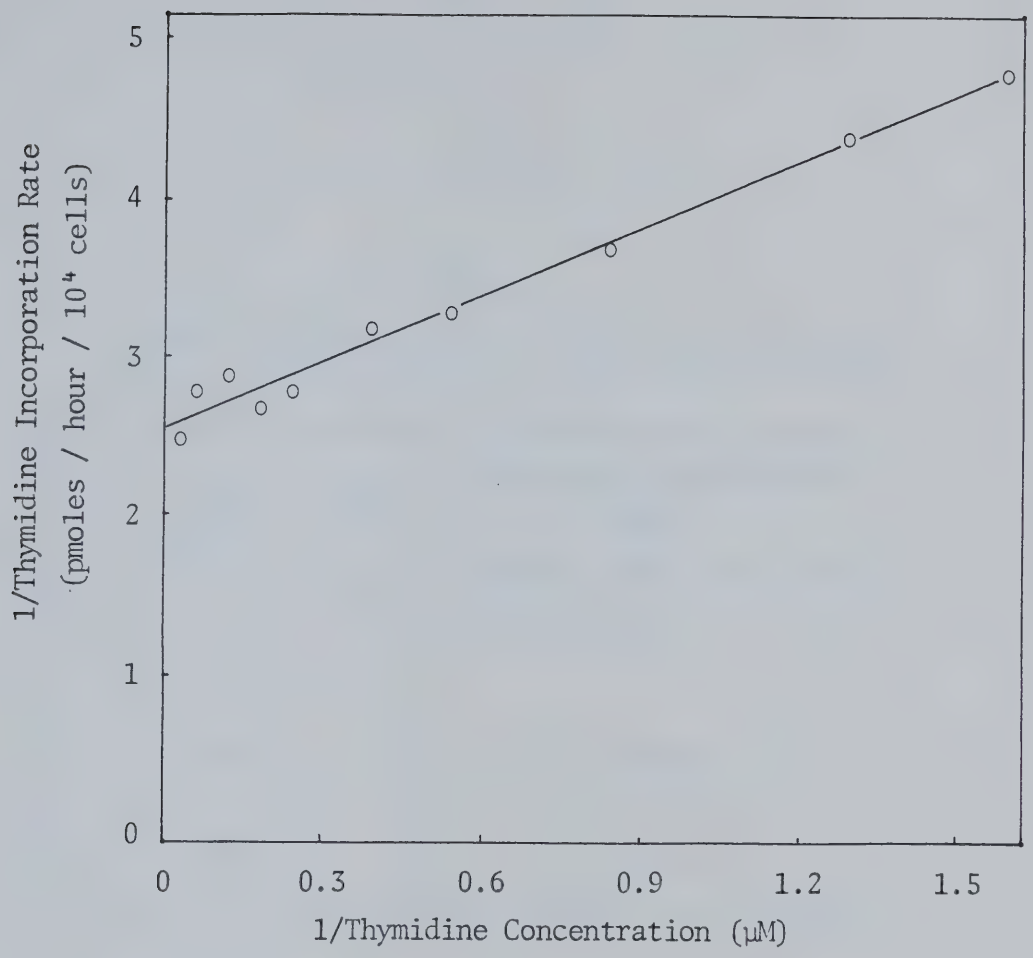


Figure 1. Thymidine saturation kinetics.

Thymocytes were cultured in 4.5 μg / ml Con A medium at 2 x 10^6 cells / ml for 48 hours. During the last 4 hours, triplicate cultures were brought to the ^{3}H-TdR concentrations indicated. The rate of thymidine incorporation was monitored for each ^{3}H-TdR concentration and expressed as pmoles / hour / 10^6 cells.

Table 1.

Thymidine Incorporation during Short Pulse Labelings.

Pulse Label Duration (minutes)	Thymidine Incorporation Rate (pmoles / hour / 10^6 cells)
15	7.36
60	7.25
120	6.65

Thymocytes were cultured at 2 x 10^6 cells / ml in 12.5 µg / ml sCon A medium for 48.5 hours. Triplicate cultures were brought to 3.4 µM ^{14}C-TdR for 15 minutes, 60 minutes or 120 minutes before harvest. The rate of thymidine incorporation into an acid insoluble form was established for each pulse labeling time.

accumulation of ^{3}H-TdR was calculated. The actual ^{3}H-TdR accumulation was monitored at 2 hour intervals in parallel cultures over the same 42 - 52 hour period. The predicted and observed accumulations of ^{3}H-TdR, in an acid insoluble form, agree for the first 8 hours of labeling (figure 2).

II.3.2 Tissue culture conditions

II.3.2.1 2-Mercaptoethanol The optimal concentration of 2-mercaptoethanol for enhancement of the thymocyte response to sCon A is 10 µM as described in figure 3a. The presence of 10 µM 2-mercaptoethanol in the culture medium resulted in a significant enhancement and quickening of the response of thymocytes to Con A (figure 3b).

II.3.2.2 Mitogens

II.3.2.2.1 Optimal mitogen concentrations The two commercial sources of Con A which were tested did not differ in the concentrations which induced optimal thymocyte stimulation (data not shown). Optimal mitogen concentrations were established by exposing cultures to a variety of mitogen concentrations (Con A 0.5 - 30 µg / ml, sCon A 1 - 100 µg / ml). The resultant stimulation of DNA synthetic activity was monitored at 48 hours. The data are presented in figure 4. The optimal Con A concentration was 6 µg / ml. The titration curve of sCon A showed a response plateau over a wide concentration range (10 - 20 µg / ml) and the toxic effects did not appear until concentrations of 20 µg / ml or greater were reached.

II.3.2.2.2 Time course of thymocyte response to mitogen

The effect of various mitogen concentrations on the time course of the thymocyte response was examined. Thymocyte cultures were exposed to Con A concentrations bracketing the optimum. The DNA

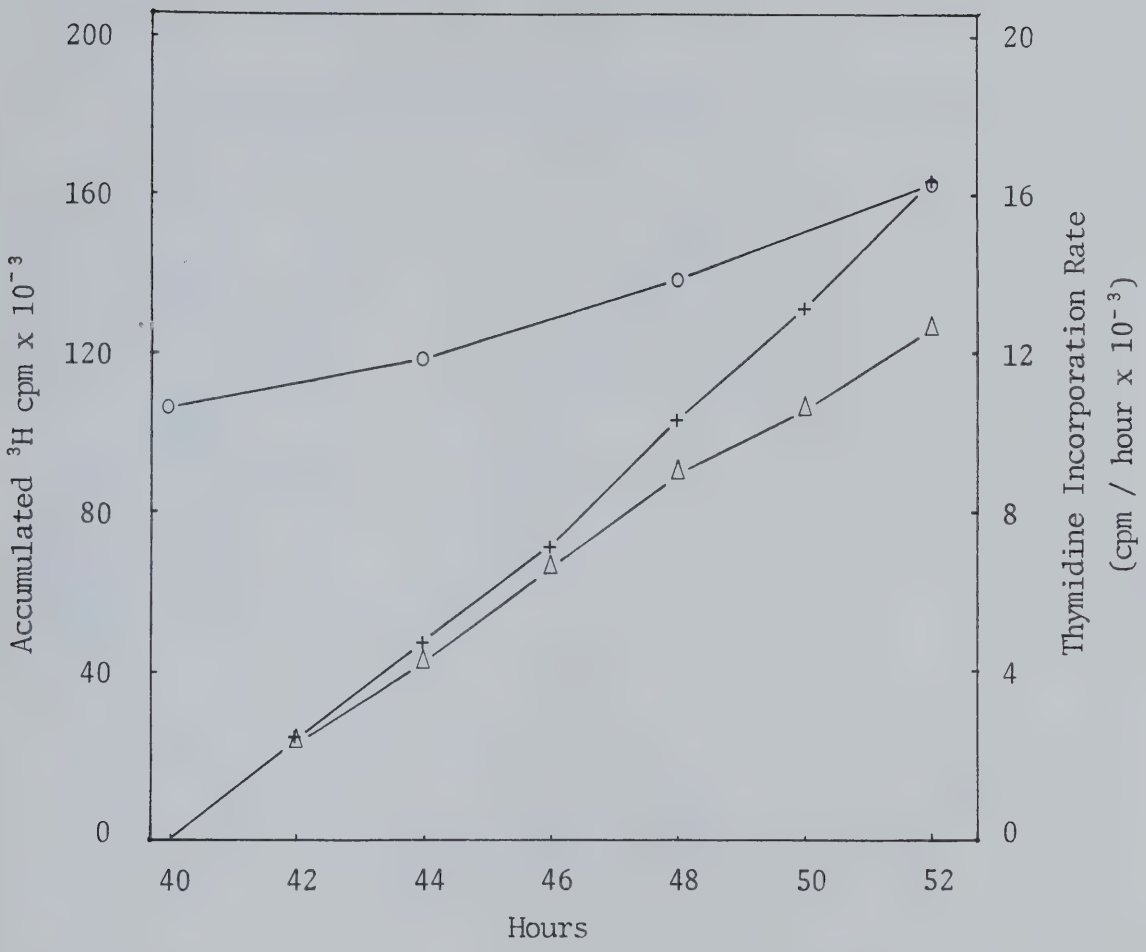


Figure 2. The linearity of thymidine incorporation into acid insoluble thymocyte material with time.

Thymocytes were cultured at 4 x 10^6 cells / ml in 4.5 µg / ml Con A medium for 40 hours. From 40 to 52 hours, cultures were labeled with 10 µM ^{3}H-TdR, either in 2 to 3 hour pulses to determine the instantaneous incorporation rate (o), or continuously to determine the accumulated ^{3}H cpm (Δ). The predicted ^{3}H cpm accumulation (+) was determined as follows:

$$\text{Predicted } ^{3}\text{H-TdR cpm accumulation at time } t = \left[\left(\frac{\text{Incorporation rate at } t - \text{Incorporation rate at } t_0}{2}\right) + \text{Incorporation rate at } t_0\right] \times t$$

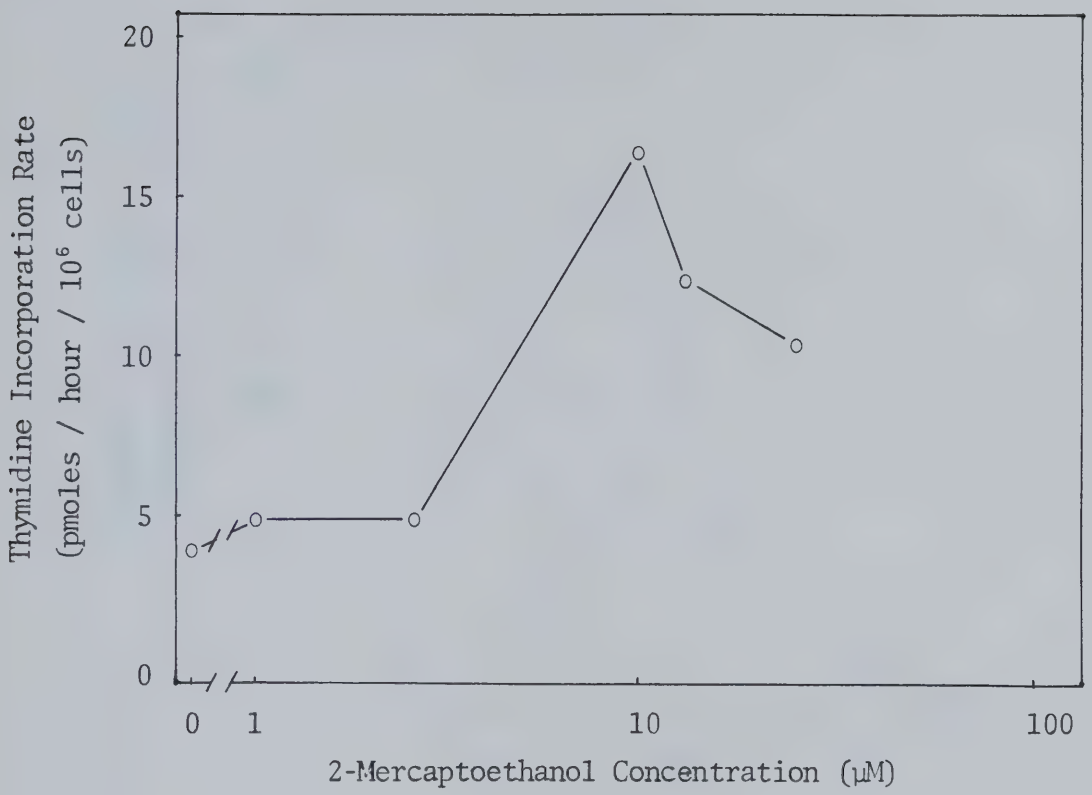


Figure 3a. The optimal concentration of 2-Mercaptoethanol for enhancement of the thymocyte response to sCon A.

Thymocytes were cultured in triplicate at 1 x 10^6 cells / ml with 20 μg / ml sCon A and the 2-mercaptoethanol concentrations indicated. The DNA synthetic rate under each 2-mercaptoethanol concentration was monitored with a 3 hour pulse label of 3.3 μM ^{14}C-TdR at 48 hours.

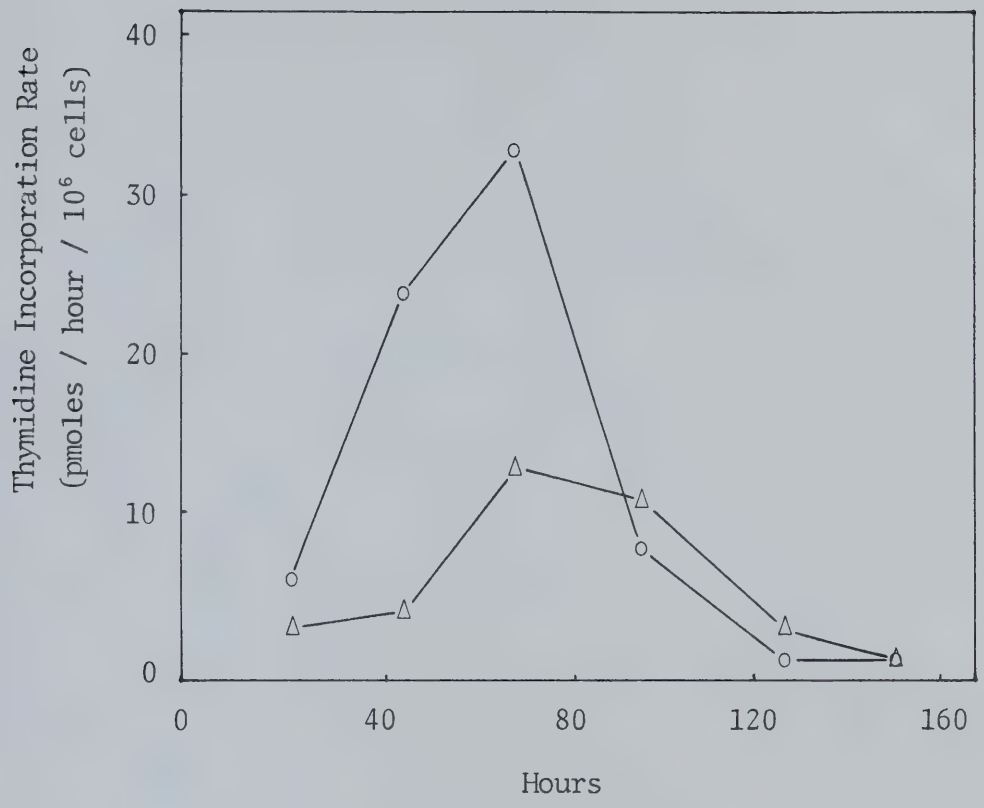


Figure 3b. The effects of 2-Mercaptoethanol on the response of thymocytes to Concanavalin A.

Thymocytes were cultured at 2 x 10^6 cells / ml in 4.5 µg / ml Con A medium in the presence (o) or in the absence (Δ) of 10 µM 2-mercaptoethanol. The rate of incorporation of thymidine into an acid insoluble form was monitored with a 3 hour pulse label of ^{3}H-TdR at the times indicated.

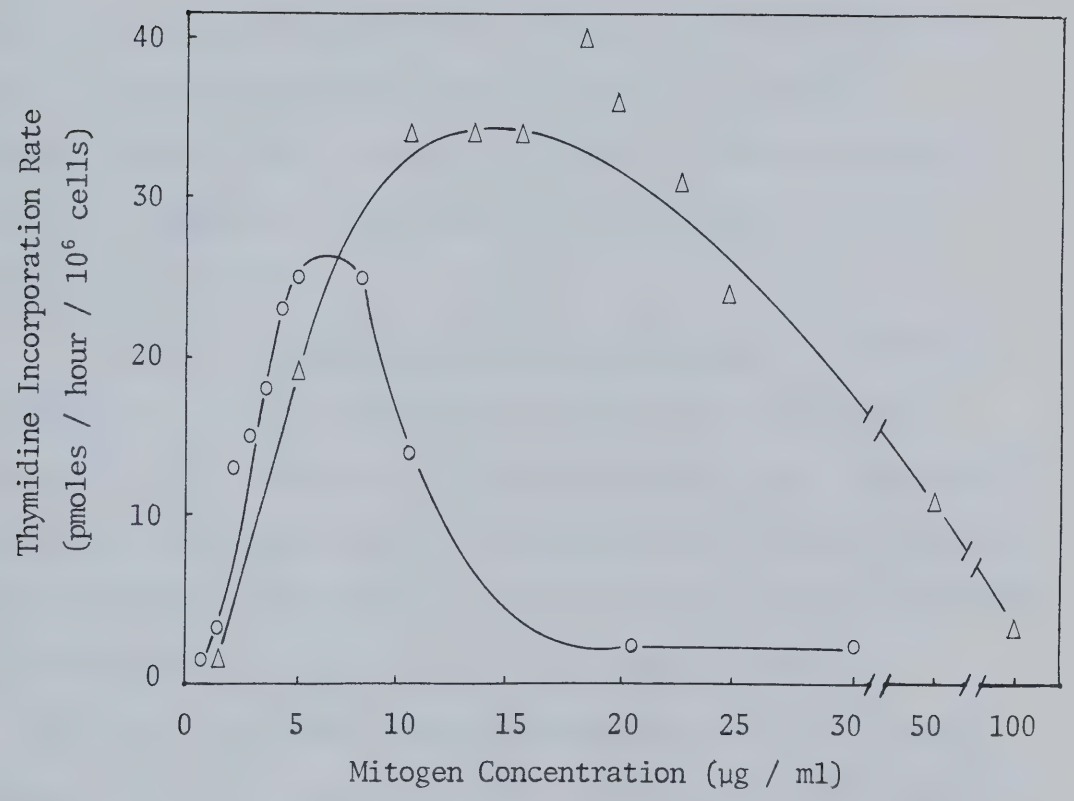


Figure 4. Optimal mitogen concentration.

Thymocytes were cultures at 1 x 10^6 cells / ml in Con A medium (○) or in sCon A medium (△) at the concentrations indicated. The rate of incorporation of thymidine into an acid insoluble form was monitored at 48 hours by a 3 hour labeling with 3.3 μM ^{14}C-TdR.

synthetic response was monitored at 24 hour intervals. The effect of increasing mitogen concentration, as seen in figure 5, was to delay the time of maximal response. Mitogen concentrations exceeding the optimum inhibited the maximal thymocyte response.

The time course of stimulation of thymocyte DNA and RNA synthesis by optimal concentrations of Con A and sCon A appear in figure 6. The time course of thymocyte response to optimal concentrations of sCon A exhibits a delayed and enhanced maximal response as compared to the response of thymocytes to optimal concentrations of Con A.

II.3.2.3 Microscopic culture properties The procedure for thymocyte isolation routinely yielded a cell suspension of greater than 90% viable cells. The centrifugation steps selected for medium and large thymocytes which comprised approximately 70% of the initial thymocyte suspension. The small thymocyte fraction was found to be unresponsive to Con A stimulation (data not shown).

The total and viable thymocyte number was found to decrease throughout the Con A response despite the presence of DNA synthetic activity. These data suggest that Con A responsive thymocytes are a short-lived, minor subpopulation of thymic tissue. As late as 120 hours, and independent of the presence of mitogen, a viable population of cells is maintained corresponding to 5 - 15% of the original number of thymocytes cultured. This subpopulation exhibits little DNA synthetic activity after 96 hours of culture.

II.3.2.4 Variation of cell density on the culture surface

Flat bottomed glass vials were substituted for the round bottomed culture tubes. A common Con A-stimulated cell stock

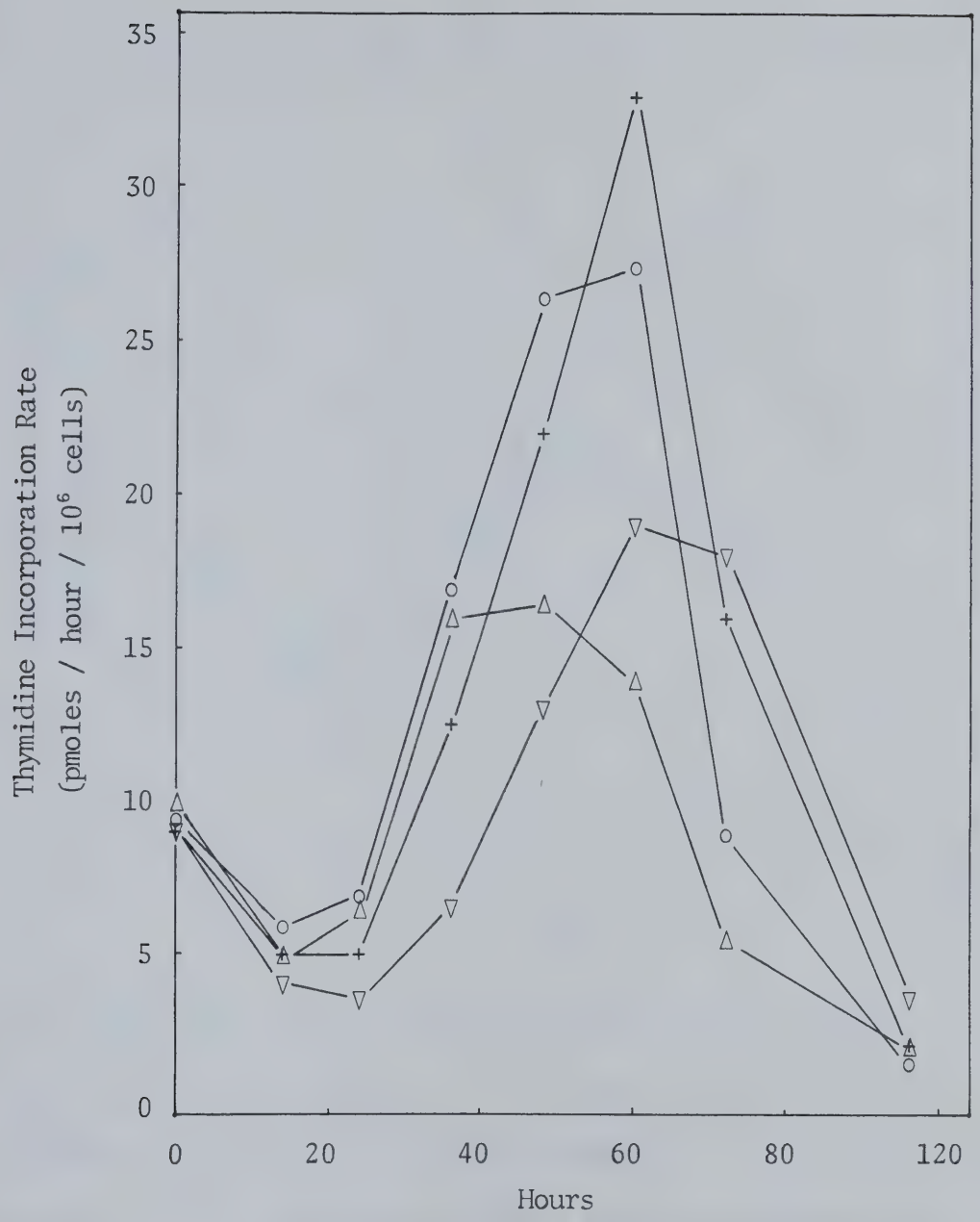


Figure 5. The effect of mitogen concentration on the time course of thymocyte stimulation.

Thymocytes were cultured at 2.5 x 10^6 cells / ml in 3.0 (Δ), 6.0 (o), 7.5 (+) or 9.0 (∇) g / ml Con A medium. At the times indicated, the rate of incorporation of thymidine into an acid insoluble form was monitored by a 3 hour, 9.5 μM ^{3}H-TdR pulse label.

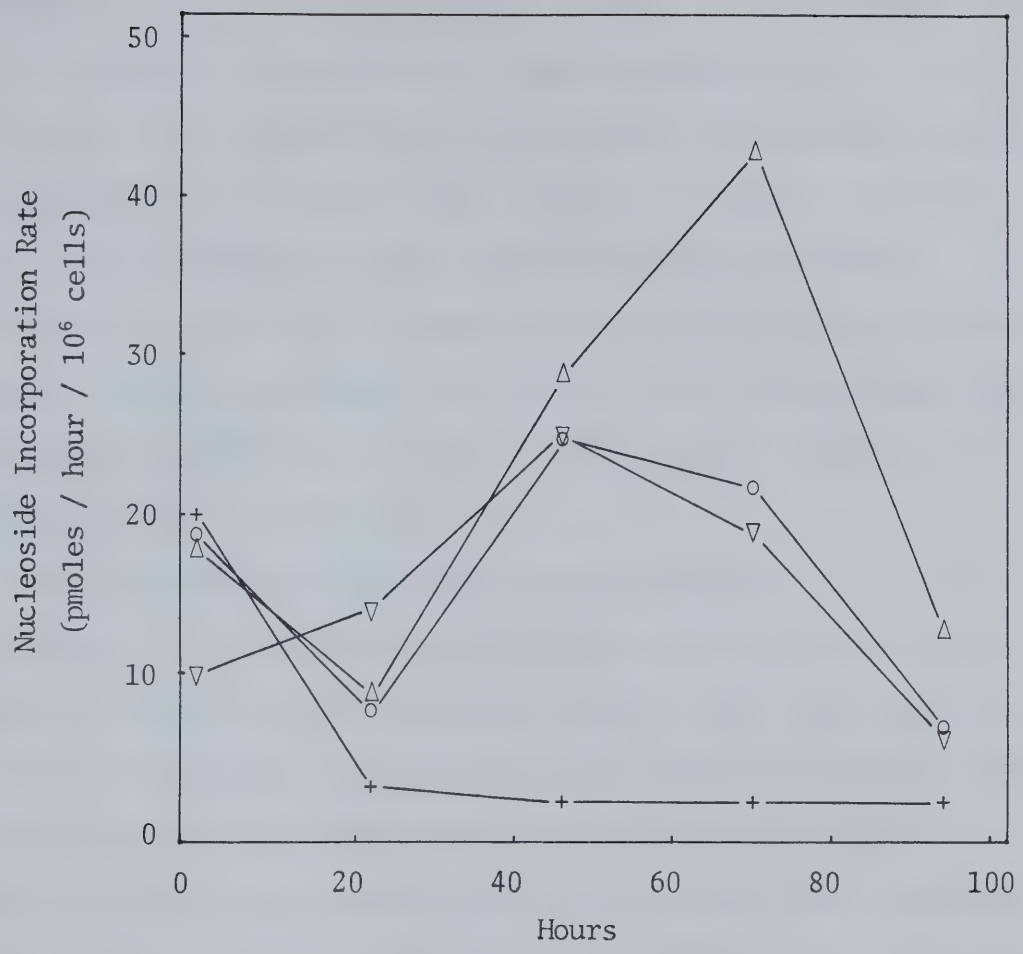


Figure 6. The time course of thymocyte stimulation by mitogens.

Thymocytes were cultured at 1 x 10^6 cells / ml in 4.5 µg / ml Con A medium (o), 12.5 g / ml sCon A medium (Δ) or in the absence of mitogen (+). At the times indicated, triplicate cultures were exposed to a 3 hour pulse label of 3.4 µM ^{14}C-TdR to establish the rate of thymidine incorporation into an acid insoluble form. Thymocytes cultured with sCon A were also pulse labeled with 12.5 µM ^{3}H-TdR to establish the rate of uridine incorporation into an acid insoluble form (∇).

containing 1.89 x 10^6 nucleated cells / ml was used to initiate 0.6 - 6.0 ml cultures. These cultures yielded densities of 0.5 - 5.0 x 10^6 cells / cm^2 of surface area. The resultant DNA synthetic activity was monitored at 12 hour intervals. The data of figure 7 indicate that cell-cell interaction may be necessary for the mitogenic stimulation of thymocytes. Cultures of low cell density fail to mount a maximal response to mitogen. The extent of cell co-operation does not appear to affect the time course of the response above an optimal level of 2 x 10^6 cells / cm^2.

Con A stimulated cultures,with average densities of 2 x 10^6, 5 x 10^6 and 1 x 10^7 nucleated cells / cm^2, were initiated in glass vials and in Diener-Marbrook culture vessels (cells were cultured on a dialysis membrane suspended in a large volume of culture medium) (Diener & Armstrong, 1969). The inhibitory effect of high cell density culturing was reduced by the more efficient Diener-Marbrook culture system (figure 8). Cultures of 1 x 10^7 cells / cm^2 failed to respond to Con A in vials. In the Diener-Marbrook system, however, cultures of 1 x 10^7 cells / cm^2 showed 60% of the response exhibited by cultures at the optimal 2 x 10^6 cells / cm^2.

II.4 Discussion

The results of this chapter have established the conditions necessary for the reproducible and optimal incorporation of exogenous thymidine into acid insoluble thymocyte material. This technique can therefore be used to monitor the rate of thymocyte DNA synthetic activity. Exogenous thymidine rapidly reaches equilibrium with the endogenous thymidine pool. This is seen in the rate of thymidine

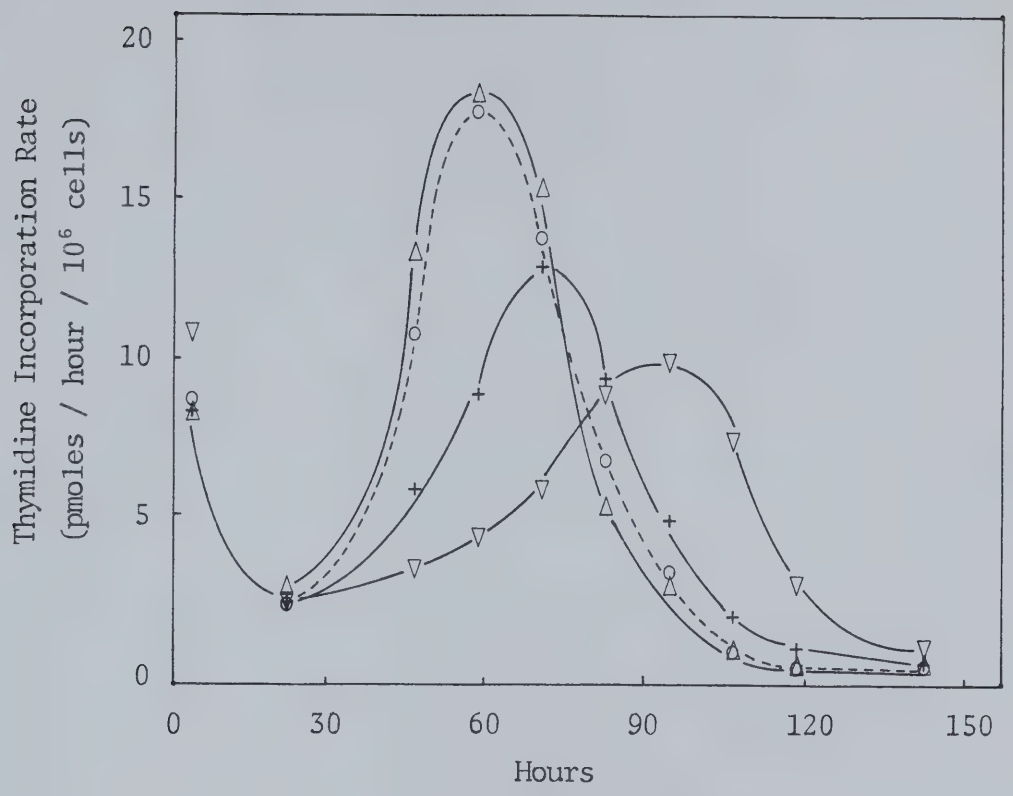
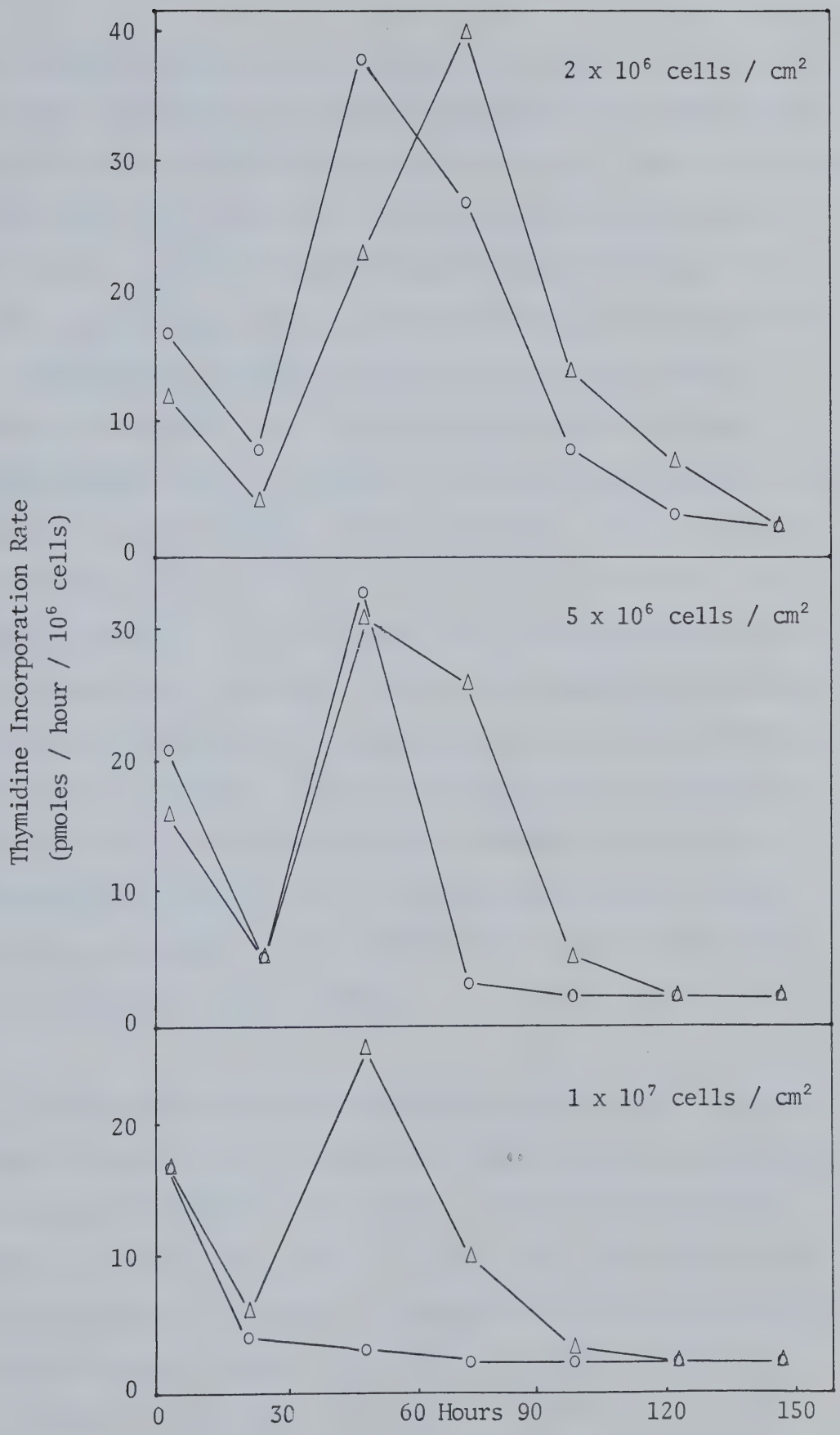


Figure 7. The effect on thymocyte stimulation of varying the cell density.

Thymocytes were cultured at 2 x 10^6 cells / ml in 7.5 µg / ml Con A medium in flat-bottomed vials. The cell number to surface area ratios were 0.5 x 10^6 / cm^2 (∇), 1.0 x 10^6 / cm^2 (+), 2.5 x 10^6 / cm^2 (o) and 5 x 10^6 / cm^2 (Δ). The thymidine incorporation rate under each culture condition was monitored by 3 hour pulse labels with 9.7 µM ^{3}H-TdR at the times indicated.

Figure 8. The effect of the Diener-Marbrook culture system on the stimulation of thymocytes at various cell concentrations.

Thymocytes were cultured in 4.5 µg / ml Con A medium in flat-bottomed glass vials (o) or in Diener-Marbrook culture vessels (Δ). The cell number to surface area ratios were 2×10^6 / cm^2, 5×10^6 / cm^2 and 1×10^7 / cm^2 with cell densities of 2×10^6 / ml, 5×10^6 / ml and 1×10^7 / ml respectively. Thymidine incorporation rates were monitored at the times indicated by 3 hour pulse labels with 4.7 µM ^{3}H-TdR in vial cultures and 18.9 µM ^{3}H-TdR in Diener-Marbrook cultures.


Thymidine Incorporation Rate
(pmoles / hour / 10⁶ cells)
2 x 10⁶ cells / cm²
5 x 10⁶ cells / cm²
1 x 10⁷ cells / cm²
40
30
20
10
0
30
20
10
0
20
10
0
0
30
60
Hours
90
120
150

incorporation exhibited by a 15 minute pulse label (table 1). The long term accumulation of exogenous thymidine occurs to the extent predicted by thymidine pulse labels up to 8 hours. These observations justify the routine use of a 2 - 4 hour pulse labeling time. The use of near saturating concentrations of thymidine during pulse labels increases the precision of DNA synthetic activity estimations.

Macromolecular synthetic activity in any living system is subject to variabibity due to many factors. Thymocytes, being primary explant tissue, are prone to variability *in vitro* due to the variations in the *in vivo* metabolic and hormonal state at the time of thymectomy. Observed synthetic activity may be expressed as a stimulation index in an attempt to reduce this variability and to allow experimental comparison. The basis for comparing experimental results in all of this work, however, is the use of the normalized DNA synthetic parameter " picomoles of thymidine incorporated per hour per million thymocytes initially cultured ". This parameter expresses DNA synthetic rates in absolute chemical terms thereby permitting comparison of experimental results even if they differ in cell numbers or labeling times.

The microscopic profile of the time course of Con A stimulated thymocytes indicates that only a minor subpopulation of thymocytes is responsive to mitogen. The constant decrease in the total and viable cell numbers throughout the response may be a result of one of the following: 1. The Con A responsive thymocytes are a population of actively proliferating cells which is too small to affect the cell numbers of the culture as a whole. 2. The Con A responsive

thymocytes are a population of proliferating cells which exhibit a very high mortality rate. The apparent loss of accumulated TdR after 8 hours of labeling (figure 2) supports the idea that a fraction of thymocytes which are responsive to mitogen die in culture. Experiments in Chapter III indicate that Con A responsive thymocytes comprise 10 - 15% of the entire population and they exhibit a growth fraction of 0.67.

The DNA synthetic pattern of Con A stimulated thymocytes exhibits an initial lag period of 24 hours, an increase in activity to a maximum at 48 - 72 hours and a return of the activity to basal levels by 96 - 120 hours (figure 6). This response pattern can be controlled to some extent as follows: Maximal DNA synthetic activity is greatest in the presence of 10 μM 2-mercaptoethanol, in the presence of optimal mitogen concentrations and at cell densities of 2×10^6 cells / cm^2 or greater. The time required to reach the point of maximal activity can be increased in the presence of mitogen concentrations which exceed the optimum concentration and at culture densities of less than 2×10^6 cells / cm^2.

The titration curve of Con A concentration is a function of the degree to which each concentration is mitogenic and toxic. Both the maximal mitogenic response of thymocytes and the toxicity of Con A towards thymocytes increases with Con A concentration (Andersson et al., 1972). The optimal concentration of Con A was found to vary by up to 100% with different lots of fetal calf serum (data not shown). Nonspecific binding of Con A to fetal calf serum reduces the amount of Con A free to associate with thymocytes (Betel & Van Der Berg, 1972).

It has not been established whether the inhibition of the thymocyte response by high concentrations of Con A is due to toxic effects on early responding cells which may cycle into the late response or if it is due to toxic effects directed against thymocytes entering the response for the first time at 48 - 60 hours (see section III.3.2.5). It has been established that the toxicity and mitogenic potency of Con A can be influenced by physical and chemical alterations of its structure (Gunther et al., 1973) as seen in the response of thymocytes to succinyl Con A (figures 4 and 6).

It was found by Dr. V. Paetkau and Dr. G. Mills (Appendix) that Con A stimulation of thymocytes is mediated by a lymphokine which they named " costimulator ". Costimulator is released by thymocytes during the first 16 hours of Con A stimulation. Reduction of costimulator levels will inhibit mitogenesis of Con A stimulated thymocytes although costimulator is not mitogenic on its own. Its production is dependent on both Thy-1 bearing cells as well as cells of the monocyte - macrophage series. Costimulator was also found to enhance the thymocyte response to PHA.

CHAPTER III

CELL CYCLING KINETICS OF CONCANAVALIN A STIMULATED THYMOCYTES

III.1 Introduction

The Con A stimulated thymocyte system described in Chapter II consists of a proliferating subpopulation within a larger, unresponsive cell population. Proliferation studies of Con A responsive thymocytes based on direct cell counts are therefore impossible. Determination of cellular proliferation from the DNA synthetic rate based on the incorporation of exogenous thymidine is impossible until one determines the cell cycling time of the Con A responsive cells and the actual numbers of the Con A responsive cells present at any one time. Autoradiographic analysis of M-phase arrested cells after ^{3}H-TdR labeling can be applied in this situation but this technique involves undesirable chemical manipulation of the proliferation mechanism.

5-Bromo-2'-deoxyuridine (BrUdR) has been employed in cell cycle analysis of other systems. Craig-Holmes & Shaw (1976) made use of the ability of BrUdR to quench fluorescence of DNA associated dyes to study cell cycle kinetics. Isolation of BrUdR-containing DNA on the basis on its increased buoyant density was used to study the conservation of the order of DNA replication in several systems (Mueller & Kajiwara, 1966; Braun & Wili, 1969; Nagata & Meselson, 1968). BrUdR was also used to study lymphocyte restimulation (Munakata & Strauss, 1972).

The same buoyant density technique was adopted in this thesis for cell cycle kinetic analysis of Con A stimulated thymocytes. An

outline of the technique appears in figure 9. One cell cycle is defined as the time required for a cell to pass from one point in S-phase to the same point in the subsequent S-phase. This is established by the time required to observe a shift of the initially incorporated ^{3}H-TdR pulse label to a hybrid density position as a result of complementary incorporation of BrUdR.

The results of this analysis establish the ability of thymocytes to recycle under Con A stimulation, the cycling time and growth fraction of Con A stimulated thymocytes. The influence of Con A on thymocytes can be removed with the competitive inhibitor α-methyl-glucopyranoside (αMG) (Gunther et al., 1974; Jones, 1973; Powell, 1970). This permits a study of the mitogen dependence of stimulated thymocytes during the course of the response. Analysis of BrUdR-containing DNA will also establish that semiconservative DNA replication rather than repair synthesis is in effect during the BrUdR labeling period. The relation between the rate of incorporation of BrUdR and the density of the resulting DNA will establish the degree of substitution of BrUdR for thymidine residues in thymocyte DNA and thus the absolute rate of thymocyte DNA synthesis.

The analysis of BrUdR-containing DNA is an appealing method for cell cycle kinetic anaylsis of the Con A stimulated thymocyte system. It must first be established however, that BrUdR is incorporated only into thymocyte DNA and that BrUdR does not alter thymocyte metabolism or DNA synthesis. Other cell systems have exhibited specific cellular changes upon exposure to BrUdR both at the metabolic level (Schubert & Jacob, 1970) and at the DNA level (Lin & Riggs, 1972; David et al., 1974).

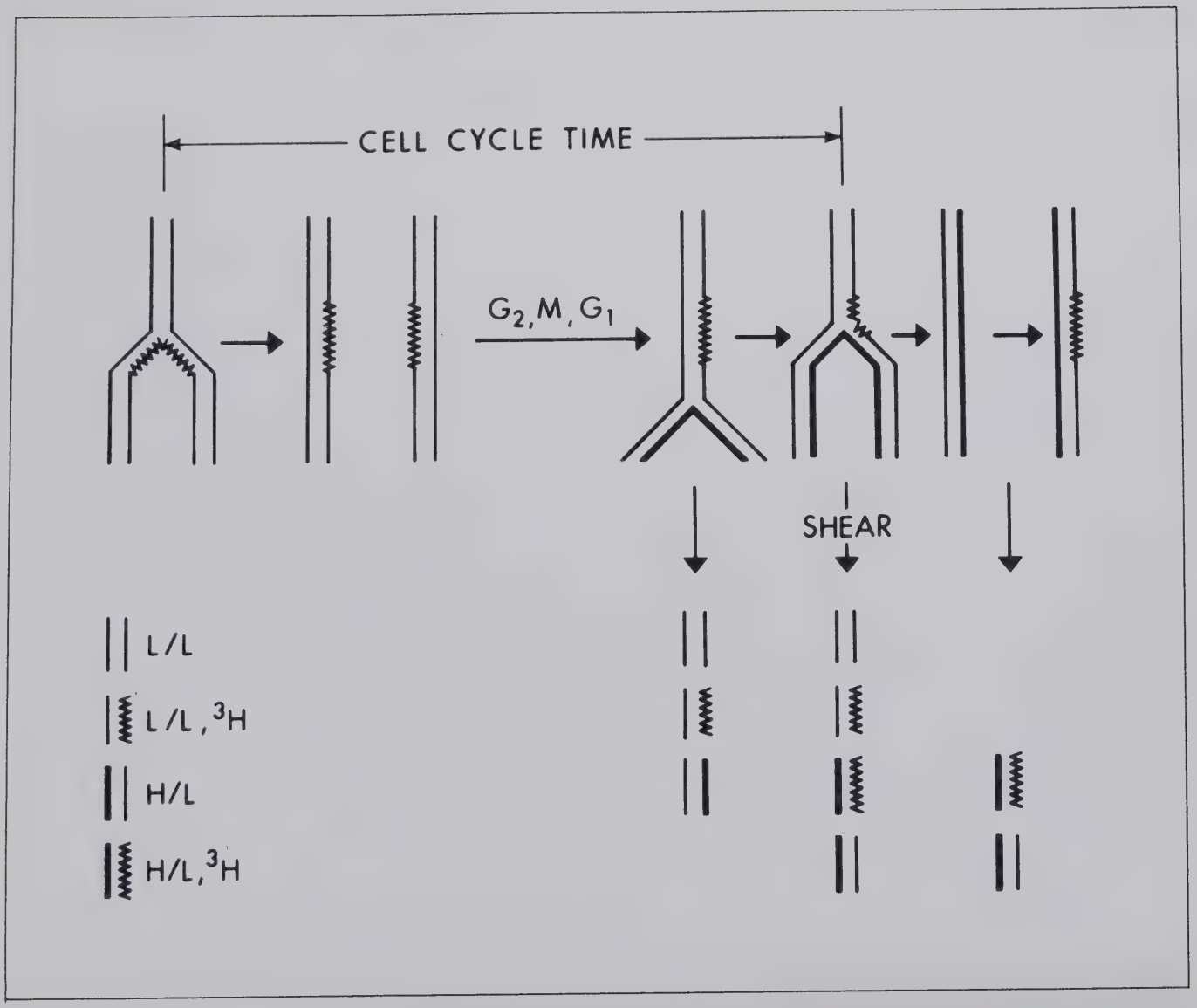

CELL CYCLE TIME
G_2, M, G_1
SHEAR
L/L
L/L, ^3H
H/L
H/L, ^3H

Figure 9. Determination of cell cycle time by the BrUdR technique.

A quantity of ^{3}H-TdR is incorporated, during a one hour labeling period followed by a wash, into thymocytes which are undergoing replication. The resulting tritiated DNA is fully low density (L/L). Addition of BrUdR results in density labeling of DNA undergoing replication. This DNA exhibits a hybrid density (H/L). Passage of the DNA which was tritiated during the ^{3}H-TdR pulse through a second S-phase in the presence of BrUdR will result in a shifting of the ^{3}H-DNA from the L/L density to the H/L density. Density analysis of short DNA fragments allows an accurate estimation of the time required for the tritiated DNA to exhibit H/L density. This time is one cell cycle - the time required to pass from a point in S-phase to the same point in the subsequent S-phase.

III.2 Methods

III.2.1 Metabolic effects of 5-bromo-2'-deoxyuridine The effect of BrUdR on the ratio of the major phosphorylated cellular macromolecules was studied as follows: Con A stimulated thymocyte cultures were brought to either 0.24 µCi ^{32}P / ml, or 0.25 µCi ^{32}P / ml plus 9.5 µM BrUdR at 22 hours. At 84 hours, appropriate cultures were pooled and centrifuged at 150 g for 5 minutes. The cell pellets were washed once with 15 ml of 0.15 M sodium chloride and resuspended in 0.5 ml of 0.15 M sodium chloride. Both suspensions received 0.1 ml of 50 mM Tris - 10 mM EDTA (pH 7), 0.2 ml of pronase (10 mg / ml heated to 80 °C for 10 minutes) and 0.2 ml of 5% sarkosyl in 50 mM Tris. Each suspension also received 0.05 ml of ^{3}H-TdR labeled T7 DNA (1.4×10^{6} cpm / ml) designed to act as an internal DNA marker. This mixture was incubated at 40 °C for several hours then vortexed. Two 0.05 ml aliquots of each sample were spotted on filter paper discs and monitored for acid insoluble ^{32}P and tritium.

Both solutions were exposed to the following organic extractions: 3 - 1 ml extractions with Tris saturated phenol - 0.1% 8-hydroxyquinoline (pH 8), 2 - 2 ml extractions with chloroform : isoamyl alcohol (24 : 1) and 2 - 2 ml ether extractions. Residual ether was removed under vacuum and both solutions were brought to 1 ml with water. Two 0.05 ml aliquots of each solution were spotted on paper filter discs and monitored for acid insoluble ^{32}P and tritium.

Both solutions received 0.1 ml of water and 0.5 ml of 1 N sodium hydroxide and were incubated at 100 °C for 5 minutes. Two 0.05 ml aliquots of each solution were spotted on paper filter discs and monitored for acid insoluble ^{32}P and tritium.

Both samples were dialysed overnight against 50 mM sodium chloride in 25 mM tris (pH 7.5). The resulting 1.2 ml samples received 0.05 ml of 5 mg / ml pancreatic DNAase, 0.07 ml of water, 0.04 ml of 1 M Tris (pH 7.5), 0.02 ml of 1 M magnesium chloride and 0.12 ml of 1 M sodium chloride. The mixtures were incubated at 37 °C and duplicate 0.1 ml samples of each were spotted on paper filter discs and monitored for acid insoluble ^{32}P and tritium after 0, 15, 30, 60 and 90 minutes of incubation.

III.2.2 Isolation of nucleic acids After incorporation of thymidine analogues, thymocyte DNA was analysed as follows: Thymocyte cultures were pooled and pelleted at 150 g for 5 minutes. The cell pellet was washed once with 10 - 15 ml of 0.15 M sodium chloride. The cell pellet was resuspended in 0.5 ml of 0.15 M sodium chloride to which was added 0.1 ml of 50 mM Tris - 10 mM EDTA (pH 7), 0.2 ml of 5% sarkosyl and 0.2 ml of 10 mg / ml pronase (heated to 80 °C for 10 minutes). The mixture was incubated at 40 °C for 3 hours. This cell lysate was exposed to 2 - 2 ml extractions with Tris saturated phenol - 0.1% 8-hydroxyquinoline (pH 8), 2 - 2 ml extractions with chloroform : isoamyl alcohol (24 : 1) and 2 - 2 ml ether extractions. The residual ether was removed under vacuum and the samples were exposed to the shearing force of vortexing, sonication or forced passage through a 26 gauge needle.

III.2.3 Density analysis of deoxyribonucleic acid Nucleic acid samples were prepared for density equilibrium centrifugation by adding cesium chloride and Tris - EDTA to establish the appropriate solution density (3.5 ml total volume). The analysis was performed in a Beckman L 265 B ultracentrifuge employing a 50 Ti fixed angle rotor.

Centrifugation was at 40,000 rpm at 15 °C for 40 - 60 hours. The resulting cesium chloride gradients were pumped from the bottom at a rate of 0.4 ml / minute onto paper filter discs as 0.1 ml fractions. The refractive index was monitored at regular intervals and related to solution density on the basis of the following relation:

Solution density = 10.87 (Refractive index) - 13.51

Filter discs were washed thrice with cold trichloroacetic acid, twice with 95% ethanol and dried. Acid insoluble radioactivity was monitored in the scintillation system described in section II.2.3. Scintillation counting data was corrected for isotope overlap where necessary and plotted as a gradient profile.

III.3 Results

III.3.1 5-Bromo-2'-deoxyuridine controls

III.3.1.1 Incorporation of 5-bromo-2'-deoxyuridine

Con A stimulated thymocyte cultures received ^{3}H-BrUdR at 46 hours. The ^{3}H-BrUdR concentration ranged from 1.04 to 10 µM. After 2 hours of labeling, cultures were monitored for acid insoluble tritium and the ^{3}H-BrUdR incorporation rate was calculated for each ^{3}H-BrUdR concentration. The results (figure 10) indicate that simple saturation kinetics are in effect during BrUdR incorporation.

III.3.1.2 Culture effects of 5-bromo-2'-deoxyuridine

Con A stimulated thymocyte cultures were brought to 4 µM ^{3}H-TdR or 4 µM BrUdR plus ^{3}H-TdR at 20 hours. Culturing was continued and samples were monitored for incorporation of tritium into an acid insoluble form at 12 hour intervals throughout the response. The long term incorporation patterns of BrUdR and TdR into acid insoluble

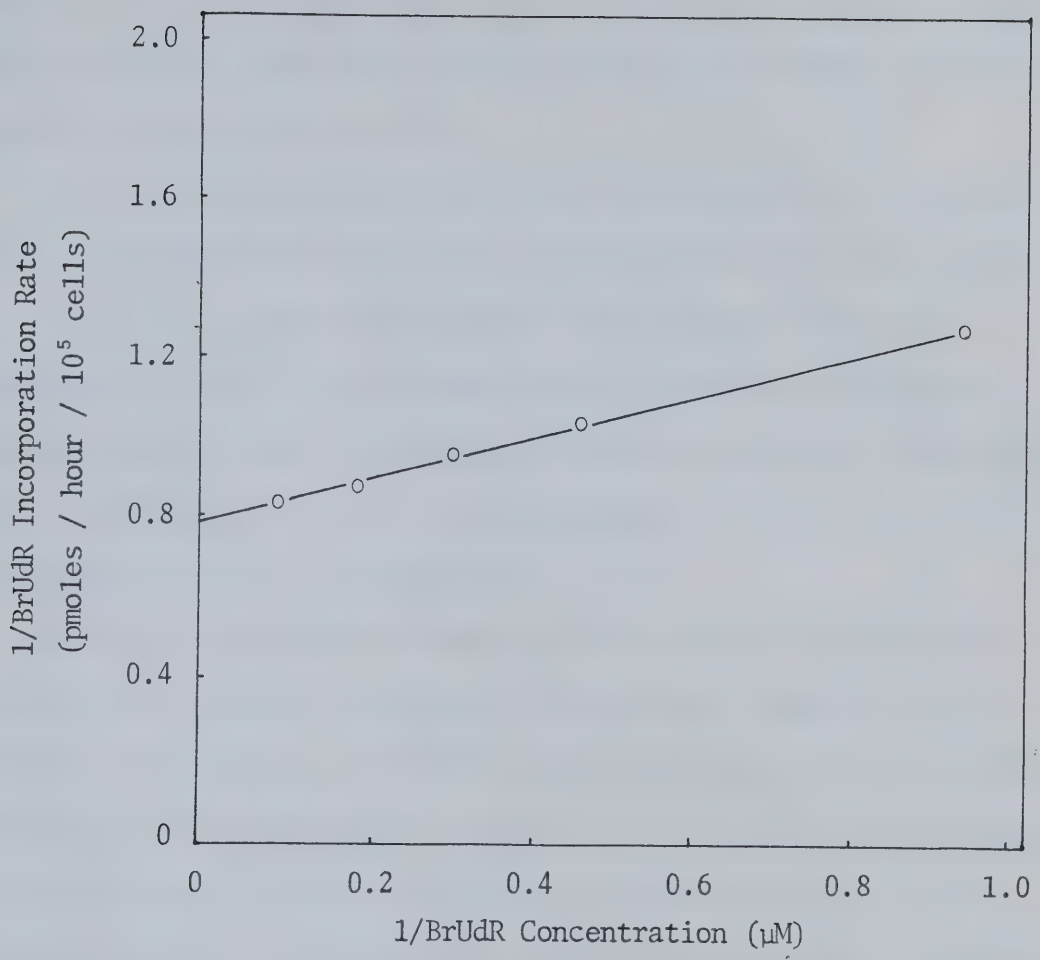


Figure 10. 5-Bromo-2'-deoxyuridine saturation kinetics.

Thymocytes were cultured in 7.5 μg / ml Con A medium at 2 x 10^6 cells / ml for 48 hours. During the last two hours, cultures were brought to the ^{3}H-BrUdR concentrations indicated. The rate of incorporation of BrUdR into an acid insoluble form was monitored for each BrUdR concentration and expressed as pmoles / hour / 10^6 cells.

material during the DNA synthetic response of thymocytes to Con A follow a similar time course as shown in figure 11a. During the course of this experiment, the BrUdR cultures exhibited 50% fewer viable cells than TdR cultures after 60 hours.

Con A stimulated thymocyte cultures were brought to 10 μM ^{3}H-TdR or 10.3 μM ^{3}H-BrUdR for three hours. These pulse labels were employed at 12 or 24 hours intervals throughout the response. Figure 11b demonstrates that the DNA synthetic response pattern of thymocytes follows a parallel time course when monitored with BrUdR or TdR pulse labels. At 60 hours, the rate of incorporation of BrUdR is 1.5 times greater than the rate of incorporation of TdR.

The effects of BrUdR on thymocyte DNA stability was studied as follows: Con A stimulated thymocyte cultures were incubated with 42 nM ^{3}H-TdR. At 38 hours, the ^{3}H-TdR medium was replaced with fresh Con A medium and the cultures were brought to 4.1 μM BrUdR. The retention of acid insoluble ^{3}H-TdR per culture was monitored at 12 or 24 hour intervals. Figure 12 indicates that the presence of BrUdR in culture has no effect on the stability of thymocyte DNA in terms of the retention of acid insoluble ^{3}H-TdR.

III.3.1.3 Metabolic effects of 5-bromo-2'-deoxyuridine

Incorporation of ^{32}P into phosphorylated macromolecules was examined as a crude monitor of general metabolic activity. Con A stimulated thymocyte cultures were brought to 0.47 μCi ^{32}P / ml or 0.47 μCi ^{32}P / ml plus 4 μM BrUdR at 30 hours. Culturing was resumed and acid insoluble ^{32}P incorporation was monitored at 10 hour intervals. Figure 13 indicates that incorporation of ^{32}P into acid insoluble material is not affected by the presence of BrUdR.

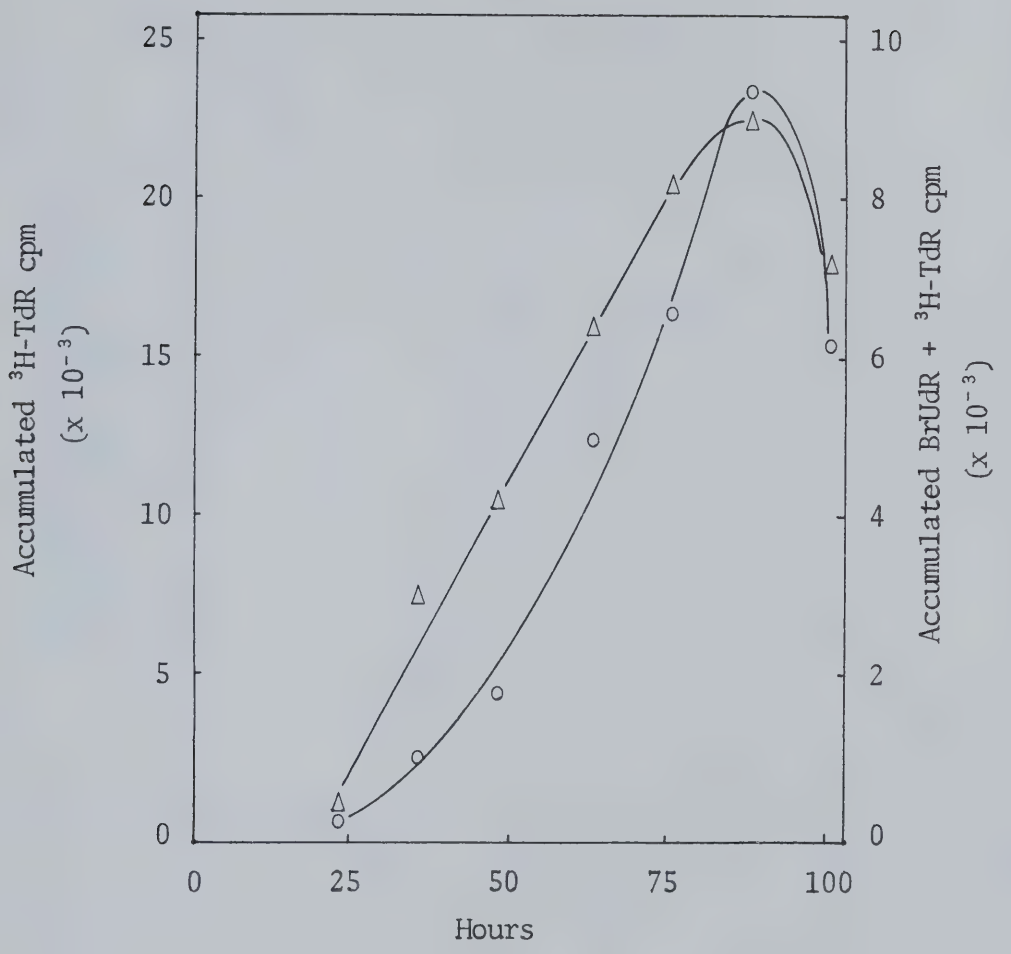


Figure 11a. The culture effects of 5-Bromo-2'-deoxyuridine - Long term label incorporation.

Thymocytes were cultured in 4.5 µg / ml Con A medium at 2 x 10^{6} cells / ml. At 21 hours, cultures were brought to 4 µM TdR + 4 nM ^{3}H-TdR (o) or 4 µM BrUdR + 4nM ^{3}H-TdR (Δ). The accumulation of tritium in acid insoluble thymocyte material was monitored at the times indicated.

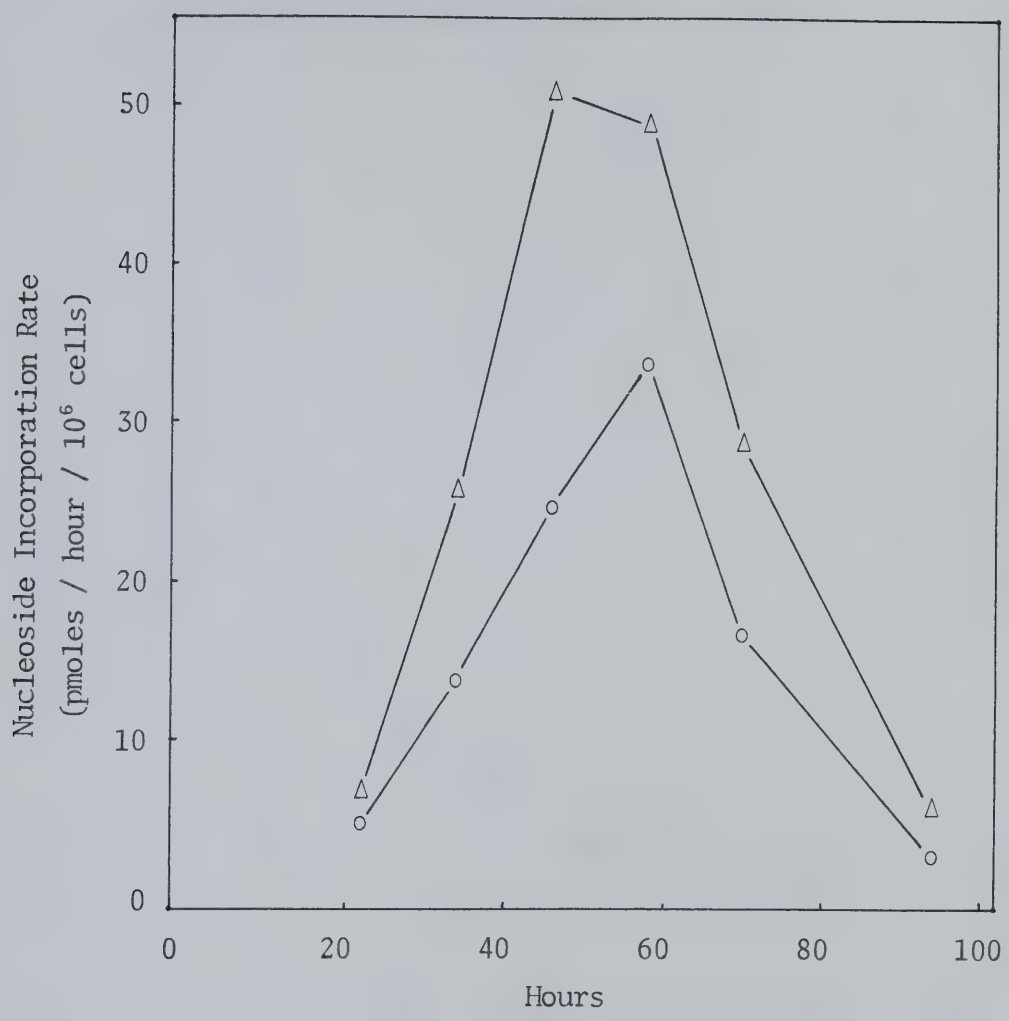


Figure 11b. The culture effects of 5-Bromo-2'-deoxyuridine - Short term label incorporation.

Thymocytes were cultured in 7.5 µg / ml Con A medium at 2 x 10^6 cells / ml. At the times indicated, the rate of nucleoside incorporation into acid insoluble material was established using 3 hour pulse labels of 10 µM ^{3}H-TdR (o) or 10.3 µM ^{3}H-BrUdR (Δ).

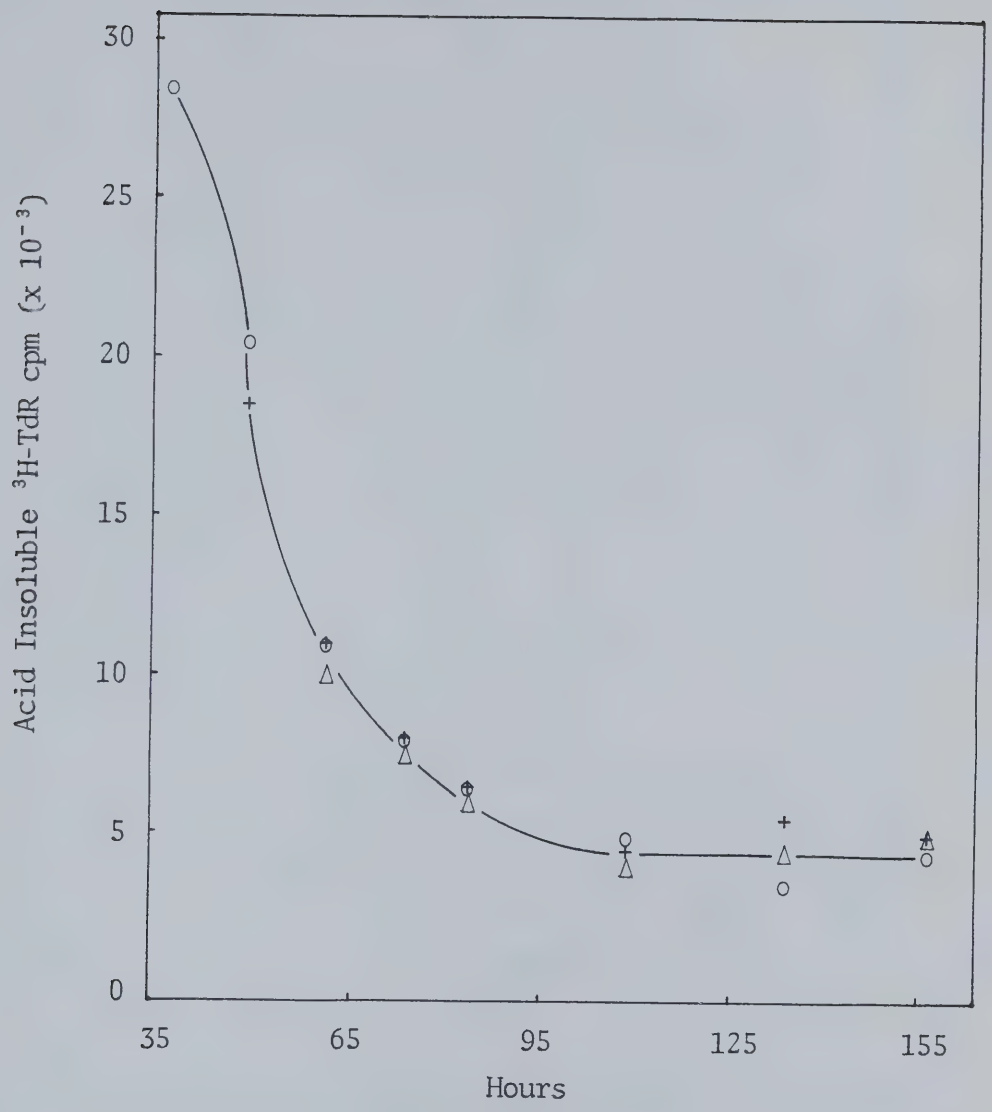


Figure 12. The effects of 5-Bromo-2'-deoxyuridine on thymocyte DNA stability.

Thymocytes were cultured in 4.5 µg / ml Con A medium at 2.3 x 10^{6} cells / ml. Thymocytes were labeled with 4.1 nM ^{3}H-TdR from 0 to 38 hours. From 38 hours onwards, cultures contained either 4.1 µM BrUdR (Δ), no BrUdR (o) or 4.1 µM BrUdR from 38 to 50 hours then no BrUdR to 156 hours (+). At the times indicated, the retention of tritium in the acid insoluble form was averaged from triplicate cultures.

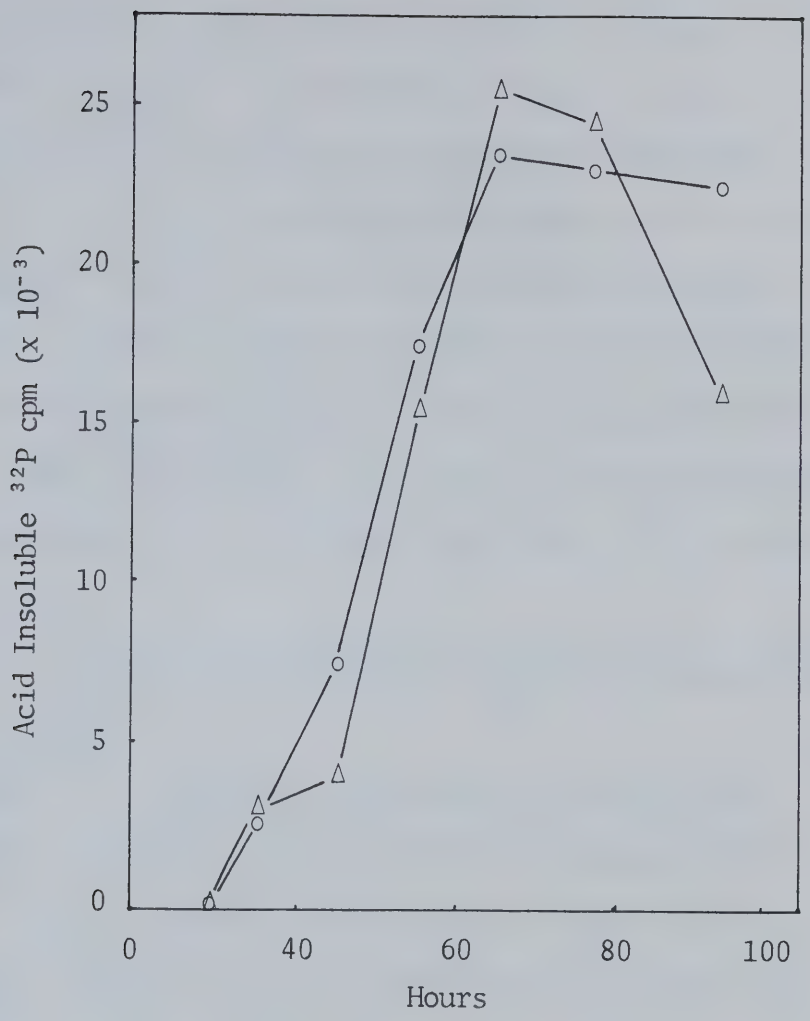


Figure 13. The effects of 5-Bromo-2'-deoxyuridine on the incorporation of ^{32}P into acid insoluble thymocyte material.

Thymocytes were cultured in 4.5 µg / ml Con A medium at 3 x 10^6 cells / ml. At 24 hours, thymocytes were isolated by centrifugation (95 g for 7 minutes) then recultured under the same conditions in fresh Con A medium. At 30 hours, cultures were brought to 0.47 µCi ^{32}P / ml (Δ) or 0.47 µCi ^{32}P / ml + 4 µM BrUdR (o). The accumulation of ^{32}P in acid insoluble thymocyte material was established at the times indicated.

The data displayed in table 2 indicates that the presence of BrUdR does not alter the ratio of the different classes of acid insoluble phosphorylated molecules within Con A stimulated thymocytes (Methods section III.2.1). Pronase treatment solubilizes most acid insoluble protein. Organic extraction removes lipid material from the cell lysate. Alkali treatment solubilizes ribonucleic acid and DNAase treatment solubilizes deoxyribonucleic acid. It is evident that the presence of BrUdR does not alter the relative amounts of phosphorylated material associated with the protein, lipid, RNA or DNA fractions of thymocyte lysates.

III.3.2 Applications of 5-bromo-2'-deoxyuridine to the study of cell cycle kinetics

III.3.2.1 5-Bromo-2'-deoxyuridine-containing DNA An example of the effect of incorporation of BrUdR into thymocyte DNA on equilibrium density centrifugation analysis is shown in figure 14. Incorporation of BrUdR into one strand of the DNA double helix yields a hybrid (H/L) density of 25 - 30 mg / cm^3 greater than the native (L/L) thymocyte DNA. Incorporation of BrUdR into both strands of the DNA double helix yields a maximal (H/H) density of 50 - 55 mg / cm^3 greater than native DNA.

Investigation of whether BrUdR was being incorporated into thymocyte DNA semiconservatively was performed as follows: The cell lysate of the 40 - 50 hour ^{14}C-TdR labeled sample of section III.3.2.2 was halved. One half was prepared for density equilibrium analysis as described. The second half was combined with Tris - EDTA (total volume of 4 ml) and denatured at 95 °C for 5 minutes. The cesium chloride was added and the equilibrium density analysis was performed.

Thymocytes were cultured at 2.2×10^6 cells / ml in 4.5 µg / ml Con A medium. At 22 hours, cultures were brought to either 0.25 µCi ^{32}P / ml + 9.5 µM BrUdR or 2.4 µCi ^{32}P / ml alone. At 84 hours, thymocytes were isolated from cultures of each label, washed once in 0.15 M sodium chloride then suspended in 0.5 ml of sodium chloride. These suspensions received 0.05 ml of 1.4×10^6 cpm / ml ^{3}H-labeled T7 DNA to act as an internal DNA standard. The suspensions were lysed and exposed to the fractionation procedures of section III.2.1. The data which appear in parentheses indicate the fraction of the acid insoluble ^{32}P or ^{3}H cpm remaining after each sample treatment.

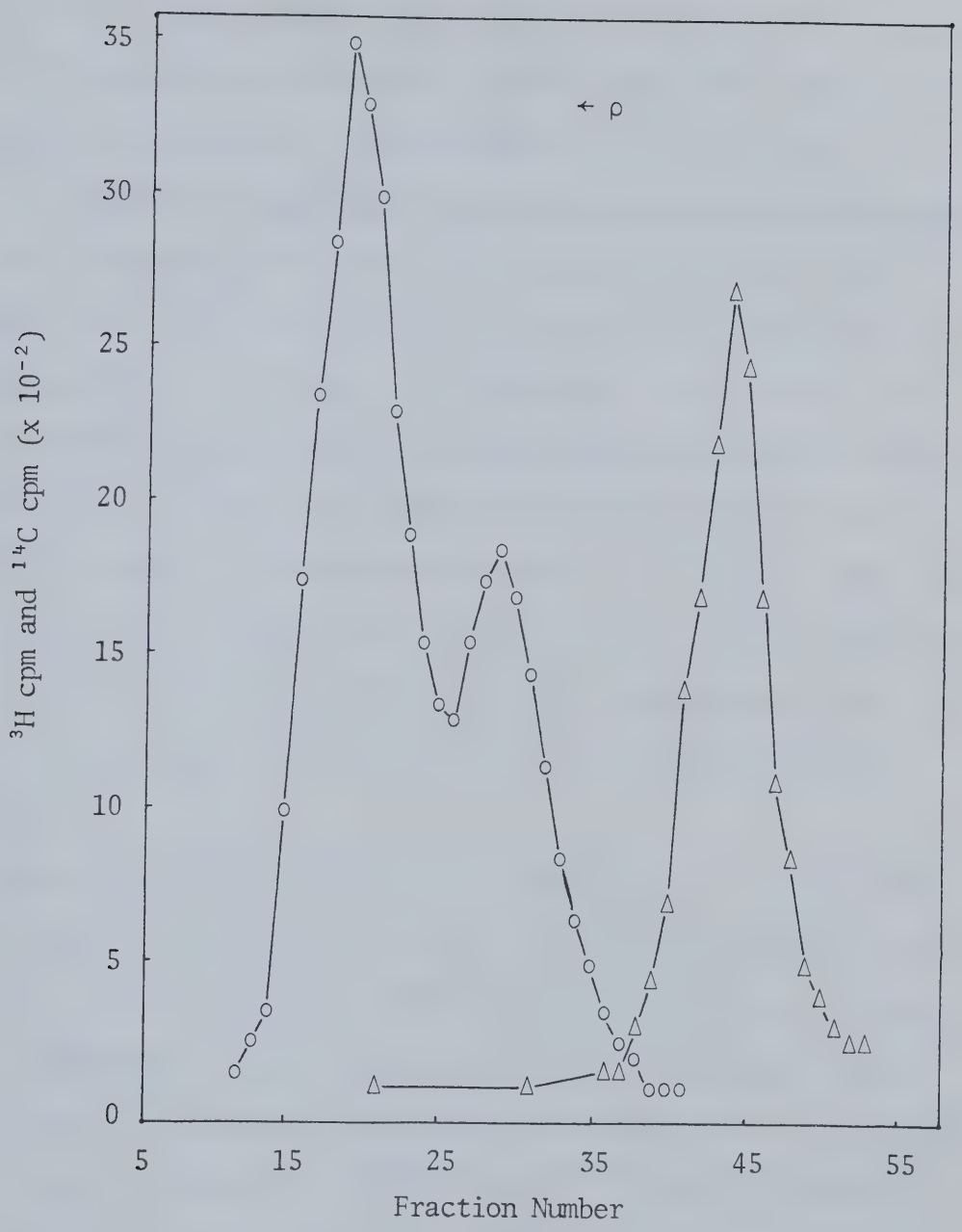
Table 2.

5-Bromo-2'-deoxyuridine Effects on Phosphorylated Macromolecules.

Sample Treatment	^{32}P + BrUdR Labels		^{32}P Label Alone	
	^{32}P cpm	^{3}H cpm	^{32}P cpm	^{3}H cpm
Pronase	38,800 (1.00)	30,880 (1.00)	50,180 (1.00)	29,290 (1.00)
Organic Extraction	23,478 (0.60)	18,438 (0.60)	31,573 (0.63)	19,677 (0.67)
Alkali	16,503 (0.43)	19,460 (0.63)	21,578 (0.43)	17,640 (0.60)
DNAase 0 min	13,585 (0.35)	2,584 (0.08)	12,350 (0.25)	1,710 (0.06)
15 min	12,084 (0.31)	1,805 (0.06)	10,488 (0.21)	1,121 (0.04)
30 min	11,134 (0.29)	2,166 (0.07)	12,065 (0.24)	1,900 (0.06)
45 min	8,816 (0.23)	912 (0.03)	11,894 (0.24)	1,786 (0.06)
60 min	10,982 (0.28)	1,520 (0.05)	11,761 (0.23)	1,501 (0.05)
90 min	8,417 (0.22)	1,273 (0.04)	10,678 (0.21)	1,786 (0.06)
120 min	8,208 (0.21)	1,140 (0.04)	8,854 (0.18)	1,577 (0.05)

Figure 14. Equilibrium density centrifugation analysis of 5-Bromo-2'-deoxyuridine-containing thymocyte DNA.

Thymocytes were cultured in 4.5 µg / ml Con A medium at 3 x 10^6 cells / ml. At 45.5 hours, thymocytes were isolated by centrifugation (435 g for 5 minutes) and recultured in fresh Con A medium containing 3.75 µM BrUdR. The culture was labeled with 100 nM ^{3}H-TdR from 55.5 to 56.5 hours, then exposed to the DNA density analysis procedures of sections III.2.2 and III.2.3. The resulting cesium chloride gradient profile is expressed as acid insoluble ^{3}H cpm per fraction (o) and acid insoluble ^{14}C cpm per fraction (Δ). The ^{14}C cpm DNA was prepared separately as a native density (L/L) thymocyte DNA marker (1.700 g / cm^3). The hybrid density (H/L) ^{3}H DNA has a density of 1.727 g / cm^3 and the high density (H/H) ^{3}H DNA has a density of 1.753 g / cm^3.


35
30
25
20
15
10
5
0
← ρ
^{3}H cpm and ^{14}C cpm (x 10^{-2})
5
15
25
35
45
55
Fraction Number

The heat denaturation of the hybrid density DNA (H/L) of figure 15a yields a total separation of the BrUdR-containing single strand from the complementary light single strand (figure 15b, Δ density = 80 - 100 mg / cm^3). This indicates that BrUdR participates in semiconservative DNA replication and does not induce a significant amount of unscheduled DNA synthesis (repair synthesis) in thymocytes.

III.3.2.2 Recycling of thymocytes during Con A stimulation

Con A stimulated thymocyte cultures were divided into three aliquots, one of which was brought to 0.78 μM ^{14}C-TdR for each of the time periods 0 - 25 hours, 25 - 40 hours or 40 - 50 hours. At the end of each labeling period, the appropriate culture was centrifuged and the cell pellet was resuspended in the original volume of fresh Con A medium. Culturing was continued to 55 hours at which time all cultures were brought to 4.1 μM BrUdR and 21 nM ^{3}H-TdR. Culturing was continued to 65 hours at which time the three aliquots were subjected to the DNA isolation and analysis procedure described previously.

The data of table 3 indicates that none of the ^{14}C-TdR incorporated in the first 25 hours of culture is shifted to the hybrid density position in the 55 - 65 hour BrUdR labeling period. However, sample B, which incorporated ^{14}C-TdR from 25 - 40 hours, and sample C, which incorporated ^{14}C-TdR from 40 - 50 hours, both exhibited hybrid density ^{14}C cpm upon incorporation of BrUdR from 55 - 65 hours. This result indicates that thymocytes involved in DNA synthetic activity during the first 25 hours of culture do not participate in the Con A induced DNA synthetic activity of the 55 - 65 hour period. Some of the thymocytes responding to Con A stimulation of DNA synthetic activity

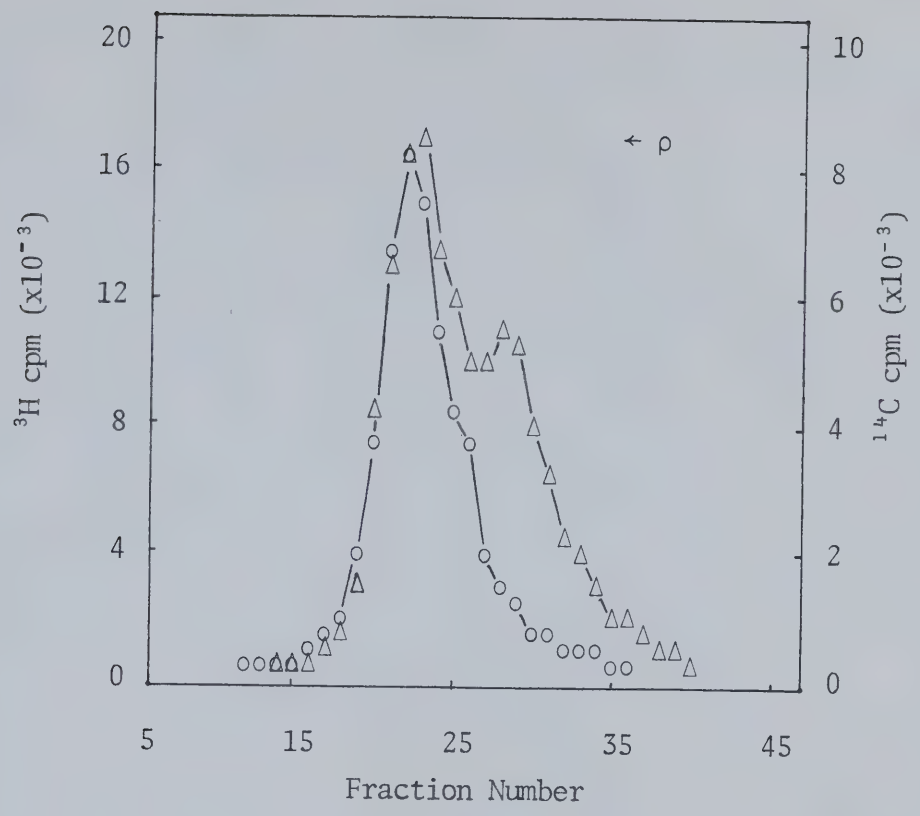


Figure 15a. Incorporation of 5-Bromo-2'-deoxyuridine into replicating thymocyte DNA - Density analysis of duplex DNA.

Thymocytes were cultured in 4.5 µg / ml Con A medium at 2.7 x 10^6 cells / ml. At 40 hours, the culture was brought to 0.78 µM ^{14}C-TdR. At 50 hours, thymocytes were isolated by centrifugation (195 g for 7 minutes) and recultured in fresh Con A medium. At 55 hours, the culture was brought to 4.1 µM BrUdR + 21 nM ^{3}H-TdR. At 65 hours, the culture was exposed to the DNA density analysis procedures of sections III.2.2 and III.2.3. The resulting cesium chloride gradient profile is expressed as acid insoluble ^{3}H cpm per fraction (o) and acid insoluble ^{14}C cpm per fraction (Δ). The native density ^{14}C-DNA appears at 1.710 g / cm^3 and the hybrid density ^{14}C and ^{3}H-DNA appear at 1.735 g / cm^3.

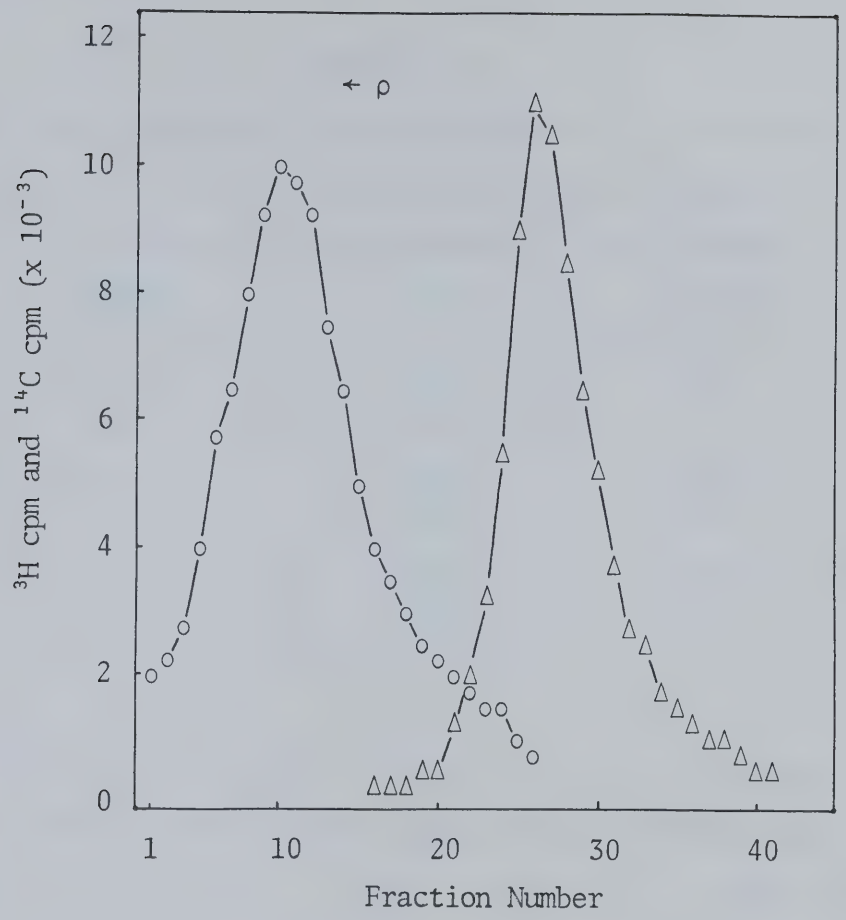


Figure 15b. Incorporation of 5-Bromo-2'-deoxyuridine into replicating thymocyte DNA - Density analysis of heat denatured DNA.

The thymocyte DNA analysed was taken from the cultures described in figure 15a. Prior to the analysis, the DNA was heat denatured (95 °C for 5 minutes) in 4 ml of 50 mM Tris - 10 mM EDTA. The cesium chloride gradient profile is expressed as acid insoluble ^{3}H cpm per fraction (o) and acid insoluble ^{14}C cpm per fraction (Δ). The native density ^{14}C-DNA appears at 1.735 g / cm^3 and the BrUdR-labeled ^{3}H-DNA appears at 1.811 g / cm^3.

Table 3.

Recycling of Thymocytes during Concanavalin A Stimulation.

	^{14}C-TdR Label (hours)	Native Density ^{14}C cpm	Hybrid Density ^{14}C cpm
A	0 - 25	9,670 (1.00)	0
B	25 - 40	22,400 (0.57)	16,900 (0.43)
C	40 - 50	29,260 (0.38)	47,740 (0.62)

Thymocytes were cultured at 2.7 x 10^6 cells / ml in 4.5 µg / ml Con A medium. Cultures were labeled with 0.78 µM ^{14}C-TdR for the time periods of 0 - 25 hours (A), 25 - 40 hours (B) or 40 - 50 hours (C). At the end of each labeling period, the thymocytes were isolated by centrifugation (195 g for 7 minutes) and recultured in fresh Con A medium. At 55 hours, all cultures were brought to 4.1 µM BrUdR and 21 nM ^{3}H-TdR. At 65 hours, all cultures were harvested and exposed to the DNA density analysis procedure of sections III.2.2 and III.2.3. The data in parentheses indicate the fraction of the total ^{14}C cpm per gradient found in each density position. Hybrid density ^{14}C cpm represents the passage of thymocyte DNA through S-phase during the ^{14}C-TdR labeling period followed by passage of the daughter DNA through S-phase during the BrUdR labeling period. This is direct evidence of thymocyte cycling during Con A stimulation.

in the 25 - 50 hour culture period do participate in the 55 - 65 hour DNA synthetic period. Thus, thymocytes can pass through at least two S-phases as a result of Con A stimulation.

III.3.2.3 Absolute amounts of recycling thymocyte DNA

Con A stimulated thymocyte cultures were labeled with ^{3}H-TdR at 35 hours. At 36 hours, the cultures were centrifuged and resuspended in half the original volume of fresh 37 °C Con A medium and divided into 8 aliquots. At 40 hours, these subcultures were brought to 4.4 µM BrUdR. Individual aliquots were harvested at 42, 44, 46, 48, 51, 55, 60 and 65 hours. After cell lysis each aliquot received an equal volume of ^{14}C-TdR labeled thymocyte DNA designed to act as an internal recovery marker. All samples were exposed to the DNA isolation and analysis procedures described previously.

The data of table 4 indicate that, of the ^{3}H-TdR-labeled DNA which was still present at 65 hours, 75% had shifted to the hybrid density position of the gradient profile upon BrUdR incorporation. Normalization of the tritium cpm with the internal ^{14}C-TdR-labeled DNA standard indicates that the absolute fraction of ^{3}H-TdR incorporated from 35 to 36 hours which was shifted to hybrid density was 0.67 after 20 hours of BrUdR labeling (figure 16 - data taken from table 4). This fraction of 0.67 is interpreted as the growth fraction of Con A stimulated thymocytes active at 35 hours. This means that 67% of the thymocytes synthesizing DNA at 35 hours have entered a subsequent S-phase by 65 hours. The remaining 33% of the thymocytes have not cycled. It is evident from the loss of total acid insoluble tritium cpm throughout the experiment (figure 16) that some thymocytes which were synthesizing DNA at 35 hours, die in culture resulting in

Thymocytes were cultured in 5.2 µg / ml Con A medium at 3.5 x 10^6 cells / ml. The cultures were labeled with 42.9 nM ^{3}H-TdR at 35 hours. At 36 hours, the thymocytes were isolated by centrifugation (150 g for 5 minutes at 37 °C) and recultured in fresh 37 °C Con A medium in 8 aliquots. At 40 hours, the subcultures were brought to 4.4 µM BrUdR. One subculture was isolated at each of the times indicated. Each sample received an aliquot of separately prepared ^{14}C-TdR-labeled thymocyte DNA and the mixture was exposed to the DNA density analysis procedures of sections III.2.2 and III.2.3. The resulting cesium chloride gradient profiles were defined in terms of acid insoluble native density ^{3}H-DNA cpm and hybrid density ^{3}H-DNA cpm. The total ^{14}C-DNA cpm was also established. The data in parentheses indicate the per centage of the total ^{3}H-DNA cpm found at the native or hybrid density positions in each gradient.

Table 4.

Absolute Amounts of Recycling Thymocyte DNA

Time from ^{3}H-TdR Exposure (hours)	Native Density ^{3}H cpm	Hybrid Density ^{3}H cpm	Total ^{14}C cpm
7	80,772 (100)	0	15,865
9	86,122 (98)	1,644 (2)	18,176
11	71,594 (90)	7,734 (10)	16,065
13	49,544 (57)	36,996 (43)	17,404
16	32,490 (40)	48,672 (60)	17,405
20	22,162 (29)	54,335 (71)	16,003
25	20,085 (26)	56,008 (74)	17,527
30	13,316 (24)	42,034 (76)	16,657

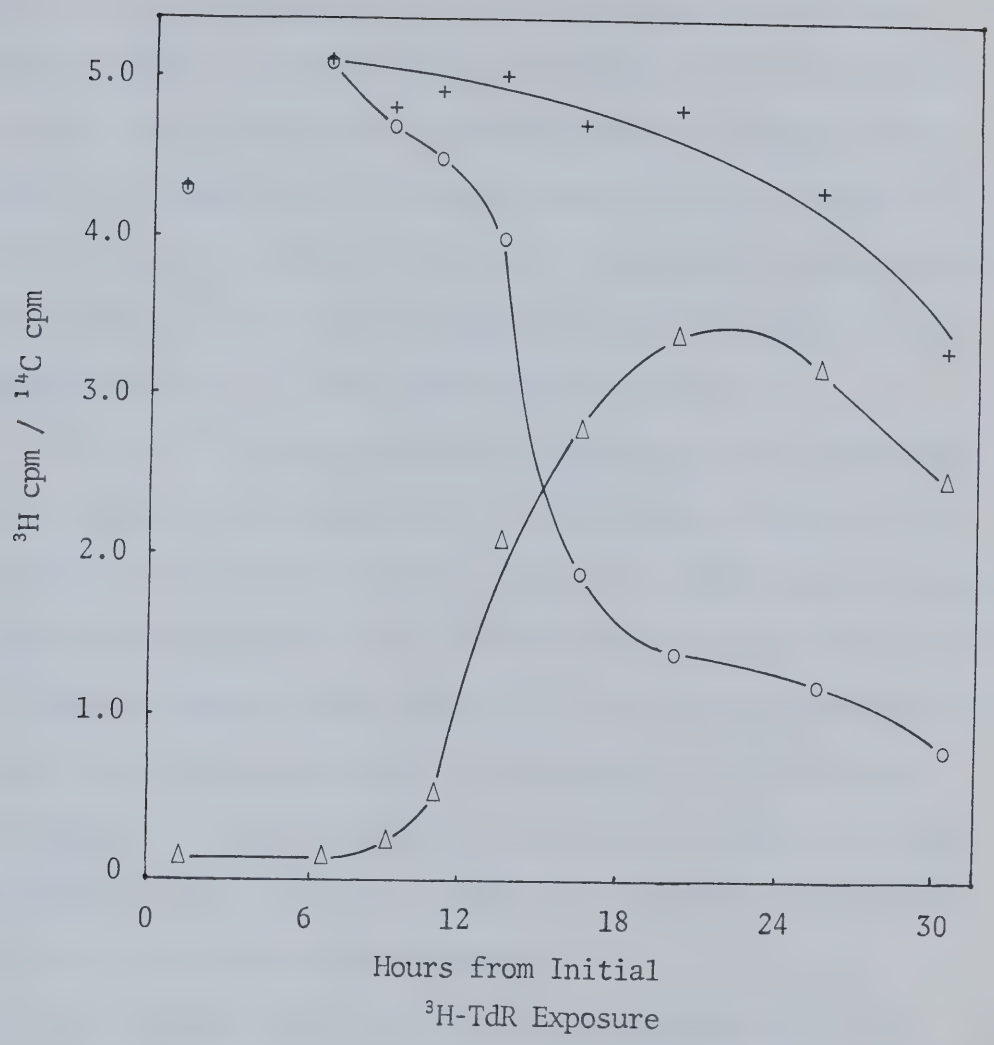


Figure 16. Absolute amounts of recycling thymocyte DNA.

This figure is a graphic representation of the data of table 4. The ratios of total ^{3}H-DNA cpm to total ^{14}C-DNA cpm (+), hybrid density ^{3}H-DNA cpm to total ^{14}C-DNA cpm (Δ) and native density ^{3}H-DNA cpm to total ^{14}C-DNA cpm (o) were calculated for each DNA sample analysed.

solubilization of their DNA.

III.3.2.4 Mean thymocyte cycling time in response to Con A

On the basis of a number of experiments comparable to that described in table 4, the mean thymocyte cycling time was found to be 12.5 ± 1.5 hours. The mean cell cycling time is defined as the time required to reach half the maximal amount of hybrid density ^{3}H cpm. The cycling time of Con A stimulated thymocytes is quite heterogeneous as seen in figure 16 (the amount of hybrid density ^{3}H cpm increases over the 9 - 25 hour BrUdR labeling period).

III.3.2.5 Thymocyte mitogen dependence Con A stimulated thymocyte cultures were brought to 3.75 μM BrUdR at 15 hours. At 25, 35 or 45 hours, 1/3 of the cultures received the following treatment: The cultures were incubated with 47 mM α-methylglucopyranoside (αMG) for 0.5 hours to competitively remove Con A from the thymocytes. The αMG-treated cells were recultured in fresh medium with BrUdR but without mitogen. At 55.5 hours all cultures were brought to 0.1 μM ^{3}H-TdR followed at 56.5 hours by exposure to the DNA isolation and analysis procedures described previously.

The DNA synthetic activity of thymocytes subsequent to the removal of Con A is described in table 5. Removal of mitogen at 25 hours resulted in an 85% inhibition of the DNA synthetic activity of thymocytes responding to Con A at 56 hours.

In this experiment, BrUdR labeling is continuous. Therefore, H/H density DNA represents participation of that DNA in at least one S-phase prior to the ^{3}H-TdR - BrUdR labeling period and the H/L density DNA represents the passage of the DNA through S-phase for the first time during the ^{3}H-TdR - BrUdR labeling period. All samples exhibited

Three samples of thymocytes were cultured in 4.5 µg / ml Con A medium at 3 x 10^6 cells / ml. All cultures were brought to 3.8 µM BrUdR at 15 hours. At 25 hours, one half of one sample was brought to 47 mM α-methylglucopyranoside (αMG) to remove Con A from the thymocytes. At 25.5 hours, the thymocytes of the αMG-treated and the control halves of the sample were isolated by centrifugation (150 g for 5 minutes). Control thymocytes were recultured in fresh Con A medium and αMG-treated thymocytes were recultured in Con A free medium. Both cultures were returned to 3.8 µM BrUdR. The identical procedure was carried out on the remaining samples at 35 and 45 hours. At 55.5 hours, all cultures were brought to 0.1 µM ^{3}H-TdR. At 56.5 hours, all cultures were harvested and exposed to the DNA density analysis procedures of sections III.2.2 and III.2.3. The resulting cesium chloride gradient profiles were defined in terms of hybrid density (H/L) and high density (H/H) acid insoluble ^{3}H cpm. High density ^{3}H-DNA represents passage of thymocyte DNA through more than one S-phase during the 15 to 56.5 hour BrUdR labeling period. Hybrid density ^{3}H-DNA represents the passage of thymocyte DNA through no more than one S-phase during the same period.

The data in parentheses represent the fraction of the total ^{3}H cpm found in the hybrid or high density position of each gradient.

Table 5.

The Mitogen Dependence of Thymocytes

Control Culture Wash Times (hours)	Hybrid Density ^{3}H cpm	High Density ^{3}H cpm
25	10,720 (0.27)	28,980 (0.73)
35	14,840 (0.35)	27,560 (0.65)
45	12,280 (0.31)	27,320 (0.69)
Con A Removal Times (hours)		
25	1,710 (0.29)	4,190 (0.71)
35	5,100 (0.28)	13,100 (0.72)
45	10,600 (0.33)	21,500 (0.67)

an average ratio of H/H to H/L density DNA of 2.25. This ratio was independent of the time at which Con A was removed or the control culture washed. These data indicate that Con A is required only to initiate a response in thymocytes. More cells are brought into the response with longer mitogen exposure but once stimulated, thymocytes are capable of proceeding through at least two S-phases in the absence of Con A. It is also evident that thymocytes can respond to Con A stimulation for the first time subsequent to the removal of Con A from the culture.

III.4 Discussion

The BrUdR controls which were performed established that BrUdR can readily be incorporated into thymocyte DNA without altering the metabolism of either DNA or other macromolecules. Incorporation of BrUdR allows measurement of the actual rate of DNA synthesis in Con A stimulated thymocytes. It has been found that substitution of BrUdR for 100% of the thymidine residues in one strand of murine DNA results in a density of 48 mg / cm^3 greater than that of native murine DNA (Rownd, 1967). In this work, the density of hybrid thymocyte DNA was found to be, on average, 29 mg / cm^3 greater than that of native DNA. Therefore, BrUdR substitutes for 60% of the thymidine residues during Con A stimulated DNA synthesis. The rate of incorporation of TdR was found to be 2/3 the rate of BrUdR incorporation, therefore, exogenous thymidine must be substituting for approximately 40% of the total thymidine residues. The rate of thymidine incorporation observed in the Con A stimulated thymocyte system is 50 pmoles / hour / 10^6 cells which corresponds to an actual rate of 125 pmoles / hour

/ 10^6 cells. Assuming a 12.5 hour cycling time, 6.5 pg of DNA per cell (Sober, 1968) and 29% thymidine content of murine DNA (Sober, 1968), the maximal rate of thymidine incorporation is 470 pmoles / hour / 10^6 cells. Thus, the observed rate of DNA synthesis is only 25% of the maximal value.

It is assumed that 3 - 12.5 hour cell cycles occur between initiation of the Con A response (24 hours) and the time of the maximal DNA synthetic response (60 hours). If the growth fraction of thymocytes in response to Con A is 0.67, then there should be $(0.67 \times 2)^3 = 2.41$ times as many Con A responsive cells at 60 hours. Since the DNA synthetic activity at 60 hours indicates that only 25% of the maximal activity is being attained, it can be assumed that only (0.25 / 2.41) x 100 = 10 - 15% of the thymocytes originally cultured were responsive to Con A.

On test of a new analytical method is its agreement with the results of other established techniques. The BrUdR technique of cell cycle analysis produced a mean thymocyte cycling time of 12.5 ± 1.5 hours which is in agreement with the reported cycling times of antigen responsive cells (13 hours - Tannenburg & Malaviya, 1968) and PHA stimulated lymphocytes (14 hours - Lohrmann et al., 1974). The results of the BrUdR technique were also in agreement with several studies (Gunther et al., 1974; Jones, 1973; Powell, 1970) reporting late involvement of mitogen in initiation of a response. Removal of Con A at 45 hours resulted in only 81% of the control DNA synthetic response at 56 hours.

The basic assumption germane to the application of the BrUdR technique to cell cycling kinetic studies of asynchronous cell populations is that DNA replication occurs in a reproducible, ordered sequence during each S-phase. Although this problem was not addressed in the thymocyte - Con A model system, the assumption generally appears valid. Replication of DNA in the early, middle and late portions of S-phase was shown to occur reproducibly in the slime mold Physarum polycephalum (Braun & Wili, 1969), in the human Hela cells (Mueller & Kajiwara, 1966) and in E. coli (Nagata & Meselson, 1968). This observation is of interest in the slime mold because the cell synchrony necessary for such a study is achieved naturally. The observation of ordered DNA replication in human Hela cells and E. coli is of interest because of the close relationship between human and murine cell systems and the fact that the order of DNA replication in both eukaryotic and prokaryotic cell systems was conserved over several cell generations. These studies are taken as evidence for the existence of a well ordered, consistent DNA replicative sequence generally.

The question of the conservation of cell cycling times from parent to daughter cells also arises from this study. Unfortunately, the BrUdR technique of cell cycle analysis is unable to answer this question directly because the cycling of a sample of DNA may be followed through no more than two subsequent S-phases. However, the observed heterogeneity of Con A stimulated thymocytes cycling times may be taken as evidence for a limited random selection of cycle times for each daughter cell. Assuming that the cycle time of each daughter cell was inherited, an experiment of 50 - 60 hours duration

would select for those cells exhibiting the shorter cycle times. In fact, the mean cell cycle time of Con A stimulated thymocytes tends to increase rather than decrease as the time of analysis is delayed (at 35 hours, thymocytes cycle in 12.5 hours; at 42 hours, thymocytes cycle in 14 hours). Therefore the observed heterogeneity of cell cycle time best fits a model of a cell population exhibiting a conserved order of DNA replication and a limited random expression of cycling times from generation to generation.

In conclusion, the BrUdR technique of cell cycle analysis proved successful in the analysis of Con A stimulated thymocytes. The technique yielded such information as the presence of cell cycling in the population, the mean cell cycling time, the heterogeneity of cycling times, the growth fraction of the cycling population and the ability of stimulated cells to cycle or to initiate a response to mitogen after removal of the mitogen. Chapter IV will discuss applications of this technique to other cell systems.

CHAPTER IV

APPLICATIONS OF THE 5-BROMO-2'-DEOXYURIDINE TECHNIQUE OF CELL CYCLE KINETIC ANALYSIS IN OTHER CELL SYSTEMS

IV.1 Introduction

The technique of monitoring cell cycle kinetics by incorporation of BrUdR was applied to two additional cell systems. It has been established (Teh & Paetkau, 1974) that an *in vitro* elevation of cyclic adenosine monophosphate (cAMP) levels in murine spleen cells during the first 12 hours of exposure to sheep red blood cells (SRBC) results in an increase of the response of the spleen cells specific to that antigen. The response of SRBC-specific spleen cells is secretion of antibody which is specific for SRBC. It became evident that the action of cAMP was to block the proliferation of a subpopulation of spleen cells responsible for the repressive control of this immune response. Supression of these repressive cells resulted in a net increase in the SRBC-specific cell number. This increase in cell number, however, could be explained by either a decrease of the doubling time of the SRBC-specific cell population to 6 - 7 hours per generation or recruitment of passive, SRBC-specific spleen cells into an active, antibody-secreting state. The answer as to which of these models was correct was sought by attempting to establish the minimum contribution of proliferation to the increase in the SRBC-specific spleen cell numbers. The BrUdR technique, which is a direct technique for studying the cell cycling of subpopulations within complex cell populations, was applied to this problem.

The BrUdR technique examines, at the DNA level, the range of cycling times of the cell population being studied. At the height of a primary immune response to SRBC, the DNA synthetic activity indicates that 1 - 5% of the viable spleen cells are synthesizing DNA. At the same time, however, only 0.1 - 0.5% of the viable spleen cells exhibit SRBC specificity. This implies that the majority of the DNA synthetic activity is not associated with spleen cells exhibiting SRBC specificity. If SRBC-specific spleen cells do have a cycling time of 6 - 7 hours, it would be difficult to resolve such data from the longer cycling time of the majority of proliferating spleen cells. However, BrUdR analysis of a spleen cell population, selected on the basis of SRBC specificity, would insure that the cycling time of SRBC-specific spleen cells would be the major contributor to the mean cell cycling time observed. Thus, the possibility that SRBC-specific spleen cells exhibit an unusually short cell cycle time could be examined in spite of the cycling of the majority of spleen cells.

The cycling of murine myeloma cells was also studied. Myeloma cells double every 16 hours. The BrUdR technique of cell cycle analysis was applied to this cell system to establish whether the cycling time obtained from BrUdR incorporation agreed with the apparent doubling time from direct cell counts of the myeloma culture.

IV.2 Methods

IV.2.1 The murine spleen cell system

IV.2.1.1 Basic culturing techniques Spleen cells were

obtained from CBA/J male mice (at least 10 weeks of age) by passage of minced spleens through fine stainless steel mesh into culture medium. The culture medium used was that of section II.2.4.1 with the concentration of 2-mercaptoethanol increased to 30 μM. The spleen cell suspension was allowed to settle for 10 minutes to remove connective tissue and cell clumps. The suspension supernatant was centrifuged at 250 g for 7 minutes. The cell pellet was resuspended in fresh medium and enumerated in terms of viable nonerythroid cells / ml on the basis of 0.15% eosin Y exclusion. The cell suspension was diluted to 1 x 10^7 viable nonerythroid cells / ml with medium, sheep red blood cells (SRBC) washed twice with medium were added to a final concentration of 0.02% and the suspension was poured into petri plates at 2 x 10^6 lymphoid cells / cm^2. N^6,$O^{2'}$-dibutyryl-adenosine-3'-5'-cyclic monophosphate (dbcAMP) was added to test plate cultures to 1 mM. After 12 hours of incubation at 37 °C, 10% carbon dioxide in air atmosphere and 100% relative humidity, the dbcAMP and control cultures were centrifuged at 400 g for 10 minutes. The cell pellets were suspended in the original volume of fresh medium and 1 ml Mishell-Dutton cultures (1967) were established in 35 mm petri plates. Cultures were returned to the original culture environment and were rocked at 3 - 4 oscillations per minute. At 24 hour intervals, each culture received 0.1 ml of the following nutritional cocktail: 1 ml of 50x essential amino acids, 0.5 ml of 100x non-essential amino acids, 0.5 ml of 200 mM glutamine, 70 mg of Eagle's Minimal Essential Medium powder, 100 mg of sodium bicarbonate, 100 mg of dextrose, 1 ml of fetal calf serum (heated to 56 °C for 30 minutes) and 7 ml of water.

IV.2.1.2 The Cunningham plaque assay At the desired times, triplicate spleen cell cultures were washed with 0.15 M sodium chloride into small plastic conical reaction tubes. The cultures were centrifuged at 400 g for 5 minutes, washed in 2 ml of 0.15 M sodium chloride and suspended in 0.5 ml of 0.15 M sodium chloride. An assay mixture was prepared consisting of 0.01 - 0.05 ml of the spleen cell suspension, 0.04 ml of washed 10% SRBC, 0.08 ml of Mishell-Dutton salts (M-D) solution (1 g of dextrose, 60 mg of potassium phosphate - monobasic, 190 mg of sodium phosphate - dibasic, 10 mg of phenol red, 400 mg of potassium chloride, 8 g of sodium chloride, 140 mg of calcium chloride, 200 mg of magnesium chloride and 83 mg of magnesium sulfate per liter of water) and 0.04 ml of 20 mg / ml guinea pig complement in M-D solution. This mixture was pipetted between two glass microscope slides, the slides were sealed with wax and incubated at 37 °C for 1 hour. Plaques or clear areas in the SRBC lawn containing a central cell as viewed under a 25x dissecting microscope were assumed to represent spleen cells producing antibody exhibiting specificity for SRBC (Cunningham & Szenberg, 1968).

IV.2.1.3 DNA synthetic activity The DNA synthetic activity associated with the immune response was monitored at 12 hour intervals by adding 0.1 ml of a 100 μM solution of ^{3}H-TdR to each plate culture 3 hours prior to the plaque-forming cell assay. The final ^{3}H-TdR concentration was 9 μM with a specific activity of 56 cpm / pmole. Incorporation of ^{3}H-TdR into acid insoluble material of aliquots of the washed spleen cell suspensions was quantified by the procedures of sections II.2.2 and II.2.3.

IV.2.1.4 Rosette formation and ficoll gradient sedimentation

Spleen cell-SRBC rosette formation was performed following the procedure of Elliot & Haskill (1973). Spleen cell cultures were pelleted and washed once in M-D solution. The number of viable cells was estimated and a 5 fold excess of M-D solution-washed SRBC was added. The suspension was centrifuged at 100 g for 5 minutes and incubated on ice for 1 hour. The pellet was resuspended and the suspension was brought to 0.6% gluteraldehyde followed by a 20 minute incubation on ice. The resulting spleen cell-SRBC rosettes were exposed to a 1 g velocity sedimentation through a ficoll gradient based on the procedure of Phillips & Miller (1970). The continuous gradient consisted of 0.35% - 1.5% ficoll in phosphate buffered sodium chloride (0.15 M) pH 7.2. The sedimentation was performed in a 10 cm diameter separation chamber at 4 °C with a total gradient volume of 500 ml. The rosette suspension was mixed with an equal volume of 0.35% ficoll then layered on the surface of the gradient. After 6 hours of sedimentation, 18 ml fractions were collected by pumping the gradient out from the bottom.

IV.2.1.5 Viable cell separation by Lymphoprep centrifugation

" Lymphoprep centrifugation " (Boyum, 1968) involves washing of the cells in Hepes buffered 0.15 M sodium chloride (pH 7.3), layering the cell suspension over 2 ml of Lymphoprep and centrifuging at 150 g for 10 minutes. Viable cells remain at the aqueous-Lymphoprep interface and the rosettes sediment to the bottom of the centrifuge tube with nonviable cells and SRBC.

IV.2.1.6 Carbonyl iron, Thy-1 and complement treatments

Carbonyl iron was employed to remove the adherent, nonantibody-

producing population of spleen cells. The spleen cell suspension was incubated with 0.6 g of carbonyl iron for 30 minutes at 37 °C. The iron and adherent cells were removed from the suspension with a strong magnet following the procedures of Lee et al. (1975).

Thy-1 serum and complement treatments were employed to remove the thymus-derived population of spleen cells which possess the Thy-1 cell surface marker. Spleen cell suspensions were incubated with a 1/5 dilution of Thy-1 serum in medium for 30 minutes at 37 °C. The mixture was diluted several fold with medium and the cells were isolated by centrifugation at 400 g for 5 minutes. The cells were resuspended in medium containing 1/6 the serum concentration of guinea pig complement and incubated at 37 °C for 30 minutes. The mixture was diluted several fold with medium and the cells were isolated by centrifugation at 400 g for 5 minutes. Viable cells were isolated by Lymphoprep centrifugation.

IV.2.1.7 Isolation of plaque-forming cells from agarose plate plaque assays Spleen cell cultures were suspended in medium containing 0.5% agarose, 2% SRBC and guinea pig complement (at 10% of the serum concentration). The suspension was poured into petri plates so that the spleen cells would occupy, on average, 1 cell / mm^2 surface area. The plates were developed at 37 °C for 2 - 8 hours and cells producing plaques in the SRBC lawn were removed from the agarose by micromanipluation. The selected cells were transfered into Hepes buffered sodium chloride containing 0.02 O.D. of calf thymus DNA to act as a carrier. The cells were released from the agarose plugs with 5 - 1 second bursts (maximum power) of a Biosonik III sonicator.

IV.2.2 The murine myeloma cell system

IV.2.2.1 Basic culturing techniques The myeloma cells employed were from the collection of the Salk Institute. They are refered to as MOPC 21 (IgG_1K) and were adapted to tissue culture (" P3 cells "). The clone used, M2, was obtained by the soft agar cloning technique 9 months prior to this study.

Myeloma cells were cultivated in spinner flask cultures using Dulbecco's Modified Essential Medium containing 42 mM Hepes buffer, pH 7.3, 100 I.U. of penicillin / ml and 10% fetal calf serum (heated to 56 °C for 30 minutes). All incubation was at 37 °C in a 100% relative humidity, 10% carbon dioxide in air atmosphere.

IV.3 Results

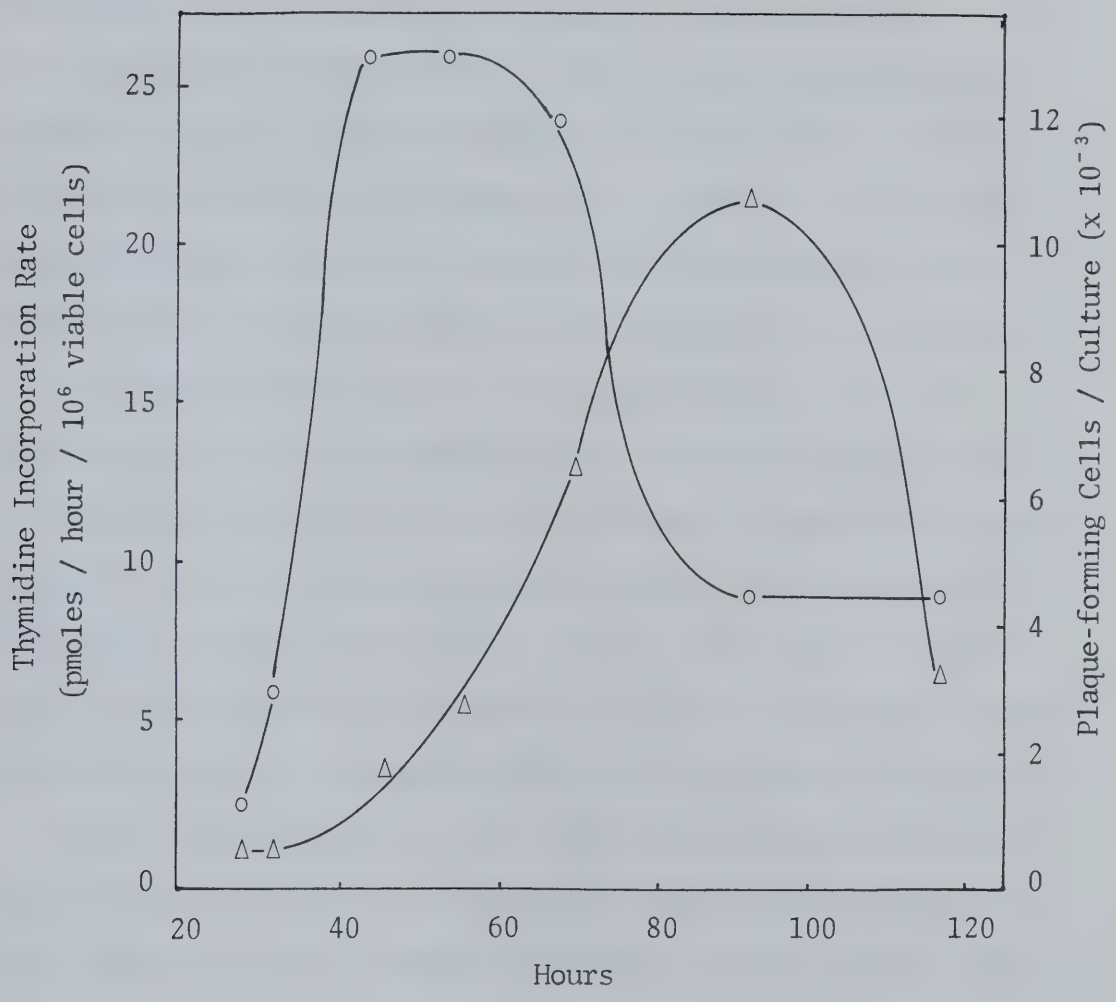
IV.3.1 The murine spleen cell system

IV.3.1.1 Analysis of the in vitro spleen cell immune response The data of figure 17 describe the time course of the response of dbcAMP-treated spleen cells to SRBC. The parameters measured were SRBC-specific plaque-forming cell numbers and the incorporation of exogenous thymidine into an acid insoluble form. It is evident that a significant period of DNA synthesis precedes the appearance of spleen cells producing antibody specific for SRBC.

IV.3.1.2 Selection for spleen cells producing antibody specific for SRBC An SRBC stimulated spleen cell culture was exposed to the techniques of rosette formation and cell sizing on a ficoll gradient (section IV.2.1.4) after 2 hours of labeling with ^{3}H-TdR. Aliquots of each fraction isolated from the ficoll gradient were counted on a model B Coulter counter. The remainder of each

Figure 17. Time course of the response of murine spleen cells to sheep red blood cells.

Spleen cells + 0.004% SRBC were exposed to 2 mM dbcAMP for 10 hours, then recultured in 1 ml aliquots of fresh medium at 1 x 10^7 spleen cells / ml. At the times indicated, quadruplicate cultures were labeled for 3 hours with 9.1 μM ^{3}H-TdR. At the end of the labeling period, the cells were isolated by centrifugation (400 g for 5 minutes), washed once in Mishell-Dutton solution and suspended in 0.5 ml of Mishell-Dutton solution. The rate of thymidine incorporation into acid insoluble material (o) was established using 0.4 ml of each suspension. The number of plaque-forming cells per culture (Δ) was established using the technique of section IV.2.1.2.


Thymidine Incorporation Rate
(pmoles / hour / 10^6 viable cells)
Plaque-forming Cells / Culture (x 10^{-3})
Hours
0
5
10
15
20
25
0
2
4
6
8
10
12
20
40
60
80
100
120

fraction was analysed for acid insoluble tritium incorporated. The ficoll gradient profile of glutaraldehyde fixed rosette-forming spleen cells appears in figure 18.

The extent of selection for viable cells, plaque-forming cells and rosettes by multiple Lymphoprep centrifugations of SRBC-stimulated spleen cells is described in table 6. Rosette formation (without glutaraldehyde fixation) by a viable spleen cell population followed by a Lymphoprep centrifugation resulted in a 5 - 6 fold selection for SRBC-specific spleen cells in terms of viable cells per plaque-forming cell. This selection for SRBC-specific spleen cells could not be enhanced by a second Lymphoprep centrifugation.

The extent of the selection for plaque-forming cells and proliferating cells by treatment of SRBC stimulated spleen cells with carbonyl iron, Thy-1 serum and complement is described in table 7. These treatments did significantly decrease the total number of viable cells in the spleen culture. However, the number of viable cells / plaque-forming cell increased, indicating that the net result was not the desired selection for SRBC-specific plaque-forming cells.

All of these attempts to select for SRBC-specific spleen cells did not result in a sufficient increase in the plaque-forming cell / total lymphocyte ratio. Therefore, analysis of SRBC-specific spleen cell cycling, using the BrUdR technique, was not possible.

IV.3.1.3 The effects of incorporation of 5-bromo-2'-deoxyuridine, high specific activity tritiated thymidine and ^{32}P on plaque formation The rate of incorporation of exogenous BrUdR and TdR into acid insoluble spleen cell material was monitored by exposing dbcAMP and SRBC-treated spleen cell cultures to ^{3}H-TdR and ^{3}H-BrUdR then

Figure 18. Ficoll gradient analysis of rosette-forming spleen cells.

Spleen cells were cultured at 1 x 10^7 cells / ml with 0.02% SRBC in the Mishell-Dutton culture system for 95 hours. At that time, cultures were labeled with 14.8 µM ^{3}H-TdR then isolated by centrifugation (435 g for 5 minutes) at 97 hours. Cells were washed once in Mishell-Dutton solution then suspended in phosphate buffered sodium chloride. The cell suspension was exposed to the rosette formation procedures of section IV.2.1.4 then layered on a 500 ml 0.35% - 1.5% ficoll gradient pH 7.2. Sedimentation at 1 g was performed at 4 °C for 6 hours then 18 ml fractions were pumped out from the bottom. Duplicate aliquots of each fraction were diluted 10 fold with 0.15 M sodium chloride then analysed with a Coulter counter in the presence of Zap Isoton (Δ). The acid insoluble thymidine incorporation of the remainder of each fraction was established (o). In spite of the presence of Zap Isoton, whole SRBC were observed in the fractions 20 through 25.

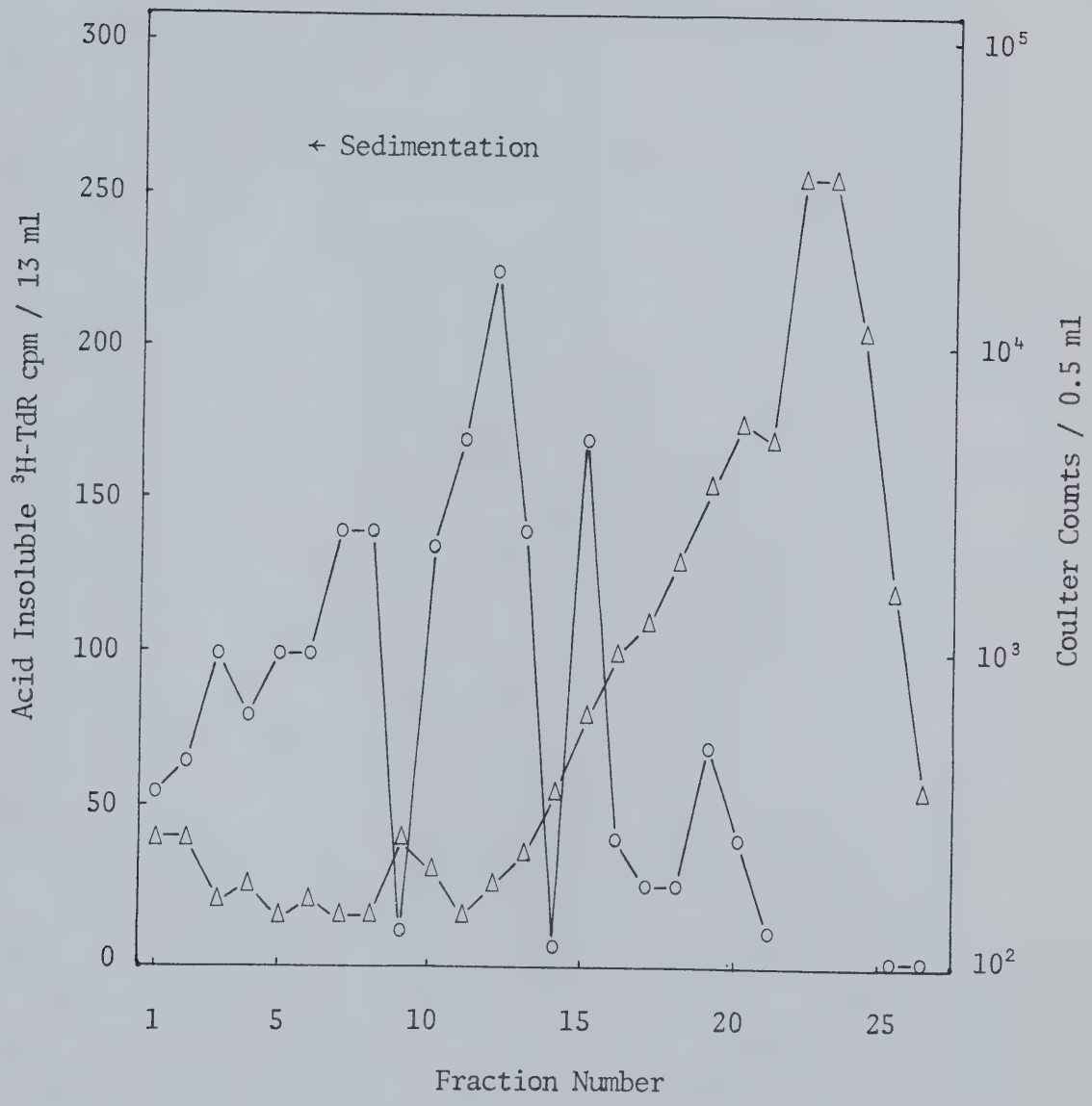

300
250
200
150
100
50
0
← Sedimentation
Acid Insoluble ³H-TdR cpm / 13 ml
10⁵
10⁴
10³
10²
Coulter Counts / 0.5 ml
1
5
10
15
20
25
Fraction Number

Table 6.

Enrichment for Rosette-forming Cells.

Sample Treatment	Viable Cells ($\times 10^{-6}$)	Rosettes ($\times 10^{-6}$)	Plaque-forming Cells	Total Cells per Plaque-forming Cell	Rosettes per Plaque-forming Cell
1. Initial Culture	20.4	-	16,350	1,250	-
2. Lymphoprep (interface)	14.8	-	16,200	916	-
Rosette Formation					
3. Lymphoprep (interface)	8.46	0	4,375	1,934	0
4. Lymphoprep (pellet)	0.15	0.06	900	233	67
Lymphoprep of Sample 4.					
5. Interface	0.04	0	200	200	0
6. Pellet	0.07	0.02	263	342	76

Spleen cells were cultured at 1 x 10^7 cells / ml with 0.02% SRBC in 0.95 M dbcAMP medium. After 12 hours, the cells were isolated by centrifugation (435 g for 7 minutes), suspended in fresh medium and cultured in the Mishell-Dutton system. At 88 hours, the cultures were pooled and total viable cell counts and plaque-forming cell counts were performed (1.). The pooled cultures were centrifuged (435 g for 7 minutes) and the cell pellet was suspended in Hepes buffered 0.15 M sodium chloride (HBS). This suspension was halved and each half was exposed to a 2 ml Lymphoprep centrifugation (2.). The viable cell suspension from the Lymphoprep interface was washed twice in fetal calf serum-free medium. SRBC were added to 0.6%. The mixture was pelleted (100 g for 5 minutes) then incubated at 4 °C. After one hour, the supernatant was removed and the cell pellet was washed once in HBS. The suspension was exposed to Lymphoprep centrifugation and the Lymphoprep pellet (SRBC and rosettes) was washed once in HBS. The Lymphoprep interface (3.) and pellet (4.) fractions were examined for viable cells and plaque-forming cells. The Lymphoprep pellet fraction (4.) was exposed to a second Lymphoprep centrifugation and the interface (5.) and pellet (6.) fractions were examined for viable cells and plaque-forming cells.

Table 7.

SRBC-specific Spleen Cell Selection using

Carbonyl Iron, Thy-1 Serum and Complement.

Sample Treatment	Viable Cells ($\times 10^{-6}$)	Plaque-forming Cells	Plaque-forming Cells per 10^6 Viable Cells	Acid Insoluble ^{3}H-TdR cpm per 10^6 Viable Cells
Initial Culture	56.9	28,445	500	35,903
Carbonyl Iron	42.2	23,709	562	27,899
Thy-1 Serum + Complement	26.9	17,400	648	37,618
Lymphoprep (interface)	17.6	12,509	710	40,722

Spleen cells were cultured at 8 x 10^6 cells / ml in 1 mM dbcAMP medium with 0.025% SRBC. After 12 hours, the cells were isolated by centrifugation (435 g for 7 minutes), suspended in fresh medium and cultured in the Mishell-Dutton system. At 67.5 hours, the cultures were brought to 0.25 μM ^{3}H-TdR. At 75 hours, the cultures were pooled and analysed for viable cells, plaque-forming cells and acid insoluble ^{3}H cpm. Cells were isolated by centrifugation and suspended in 6 ml of medium with 0.6 g of Carbonyl Iron. The mixture was incubated at 37 °C with occasional swirling. After 30 minutes, the iron was removed with a magnet and washed once with fresh medium. The combined cell suspension and wash was analysed for viable cells, plaque-forming cells and acid insoluble ^{3}H cpm. The cells were isolated by centrifugation and suspended in 0.8 ml of medium and 0.2 ml of Thy-1 serum. The mixture was incubated at 37 °C. After 30 minutes, the volume was raised to 6 ml with medium and the cells were isolated by centrifugation. The cells were suspended in 0.5 ml of medium and 0.5 ml of guinea pig complement (1/3 the serum concentration). The mixture was incubated at 37 °C. After 30 minutes, the volume was raised to 6 ml with medium and the cells were isolated by centrifugation. The cells were suspended in 1 ml of medium and analysed for viable cells, plaque-forming cells and acid insoluble ^{3}H cpm. The cell suspension was exposed to a Lymphoprep centrifugation and the interface fraction was analysed for viable cells, plaque-forming cells and acid insoluble ^{3}H cpm.

monitoring for incorporation of tritium into an acid insoluble form. The rates of incorporation were 7.3 pmoles of TdR / 10^6 viable cells / hour and 10.4 pmoles of BrUdR / 10^6 viable cells / hour.

The effects of incorporation of BrUdR on spleen cell plaque formation was studied by labeling spleen cell cultures with BrUdR and adding 2'-deoxycytidine (CdR) to half the BrUdR-treated cultures. Plaque-forming cell assays of control, BrUdR treated and BrUdR plus CdR-treated cultures were performed. The results in figure 8a indicate that incorporation of BrUdR results in a 50% inhibition of plaque formation after 24 hours. This inhibition is not at the level of cytidine metabolism (Meuth & Green, 1974) since the presence of CdR does not reverse the inhibition.

The effect of incorporation of high specific activity ^{3}H-TdR on viable cell numbers and plaque-forming cell numbers was studied as follows: SRBC-treated spleen cell cultures were labeled with ^{3}H-TdR (20 Ci / mM) in either their own conditioned medium or in fresh medium. Plaque-forming cell assays were performed on the cultures after removal of the ^{3}H-TdR from culture. The results in table 8b indicate that a 3 hour incorporation of saturating levels of high specific activity ^{3}H-TdR yields a 70% inhibition of plaque-forming ability after 12 hours and an 80% inhibition after 24 hours. It is evident that a portion of the inhibition is due to the handling of the cultures as seen from the " no handling " controls but the effect of ^{3}H-TdR itself is significant. The use of conditioned medium for reculture does not significantly reverse the inhibition.

The effect of incorporation of ^{32}P on viable cell numbers and plaque-forming cell numbers was studied as follows: A variety of ^{32}P

Table 8a.

The Effects of 5-Bromo-2'-deoxyuridine on Plaque Formation.

Culture Hours	Plaque-forming Cells per Culture		
	Control	10 μM BrUdR	10 μM BrUdR + 43.3 μM CdR
70.5	950	-	-
82.5	5,000	4,275 (85)	4,750 (95)
94	10,000	4,600 (46)	4,300 (43)

Spleen cells were cultured at 1.25 x 10^7 cells / ml in 1 mM dbcAMP medium with 0.025% SRBC. After 11 hours, the cells were isolated by centrifugation (435 g for 5 minutes), suspended in fresh medium and cultured in the Mishell-Dutton system. At 70.5 hours, some cultures were brought to 10 μM BrUdR, others were brought to 10 μM BrUdR + 43.3 μM CdR and the remaining cultures were left as controls. Triplicate cultures under each condition were analysed for plaque-forming cells at 82.5 and 94 hours. The data in parentheses indicate the per centage of the control plaque-forming cell number found in the test cultures.

Table 8b.

The Effects of High Specific Activity Tritiated Thymidine on Plaque Formation

Culture Hours	Plaque-forming Cells per Culture				
	No Handling Control	5 μM ^{3}H-TdR in Fresh Medium	Fresh Medium Handling Control	5 μM ^{3}H-TdR in Conditioned Medium	Conditioned Medium Handling Control
63	2,400	-	-	-	-
75	4,933	1,417 (29)	3,538 (72)	1,821 (37)	3,604 (73)
87	9,500	1,650 (17)	7,800 (82)	-	-

Spleen cells were cultured at 1 x 10^7 cells / ml in 1 mM dbcAMP medium with 0.02% SRBC. At 16.5 hours, the cells were isolated by centrifugation (435 g for 5 minutes), suspended in fresh medium and cultured in the Mishell-Dutton system. At 63 hours, a portion of the culture was left to act as the " no handling control " while the remainder of the cells were isolated by centrifugation (435 g for 5 minutes). These cells were suspended in either fresh medium or their own conditioned medium. Half of each suspension was brought to 5 µM ^{3}H-TdR (20 Ci / mM) while the other half acted as the " handling control ". At 66 hours, all handled cultures were increased in volume 7 fold with the appropriate medium and the cells were isolated by centrifugation (435 g for 5 minutes). The cells were cultured in the appropriate medium and triplicate cultures of each condition were analysed for plaque-forming cells at 75 anf 87 hours. The data in parentheses indicate the per centage of the " no handling control " plaque-forming cells number found in each culture.

specific activities were employed in labeling stimulated spleen cell cultures. Incorporation of ^{32}P into acid insoluble spleen cell material and plaque-forming cell analysis was performed at intervals after ^{32}P addition to the culture. The results of table 8c indicate that the incorporation of ^{32}P into spleen cells is marginally detrimental to plaque formation and does not appear to be specific activity dependent in the range tested.

IV.3.1.4 Cell cycle kinetics of spleen cells producing antibody specific for SRBC The cell cycling of dbcAMP and SRBC-treated spleen cell cultures without selection for SRBC-specific cells was studied as follows: Routine cultures were labeled with ^{3}H-BrUdR. Aliquots were harvested from 6 to 24 hours after ^{3}H-BrUdR addition. At each time point, individual cultures were prepared for the plaque-froming cell assay then pooled and prepared for equilibrium density centrifugation analysis as described in sections III.2.2 and III.2.3. The cycling pattern of dbcAMP treated whole spleen cell cultures under SRBC stimulation is found in figure 19. The mean cell cycling time is 13 hours with an apparent cycling time heterogeneity of from 9 to 19 hours. The plaque-forming cell numbers increased from 475 pfc / culture at 60 hours to 1,550 pfc / culture at 72 hours (7.4 hour doubling time).

The cell cycle analysis of SRBC-specific spleen cells was attempted as follows: Several labeling schemes were employed including initial ^{3}H-TdR pulse labeling followed by continual BrUdR labeling or initial ^{32}P labeling followed by continuous BrUdR labeling. SRBC-specific spleen cell selection was attempted using the agarose plate plaque assay technique of section IV.2.1.7. Cell cycle analysis

Table 8c.

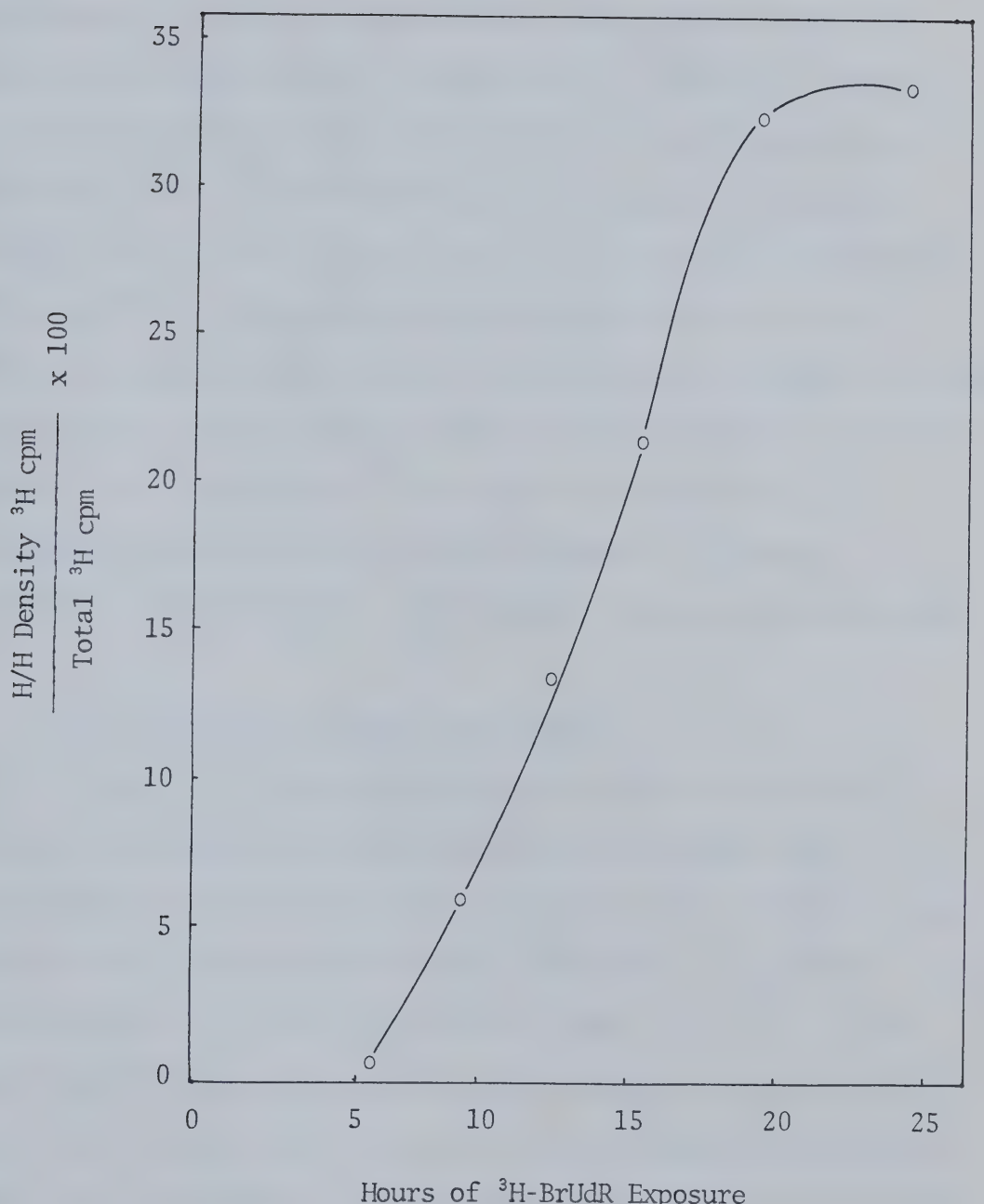
The Effects of ^{32}P on Plaque Formation.

Culture Hours	Plaque-forming Cells per Culture						
	No Handling Control	Handling Control	17 µCi ^{32}P per ml M-D	1.7 µCi ^{32}P per ml M-D	0.17 µCi ^{32}P per ml M-D	0.017 µCi ^{32}P per ml M-D	1.7 µCi ^{32}P per ml Culture Medium
60	853	-	-	-	-	-	-
72	1,113	942	642 (35,450)	900 (6,627)	783 (1,405)	758 (361)	817 (3,143)
84	1,125	1,150	883	908	642	900	1,108

Spleen cells were cultured at 9 x 10^6 cells / ml in 1 mM dbcAMP medium with 0.02% SRBC. At 13.5 hours, the cells were isolated by centrifugation (435 g for 5 minutes), suspended in fresh medium and cultured in the Mishel-Dutton system. At 60 hours, cells were isolated by centrifugation (435 g for 5 minutes) and suspended in either Mishel-Dutton solution (M-D) + 10% fetal calf serum or culture medium. These suspensions were brought to the ^{32}P activities indicated. Several medium cultures were left free of ^{32}P to act as " handling controls ". Several cultures were left untouched at 60 hours to act as " no handling controls ". The average number of plaque-forming cells in triplicate cultures of each condition was established at 72 and 84 hours. The data in parentheses indicate the average acid insoluble ^{32}P cpm of the test cultures.

Figure 19. 5-Bromo-2'-deoxyuridine analysis of whole spleen cell culture cycling.

Spleen cells were cultured at 1 x 10^7 cells / ml with 0.02% SRBC and 1 mM dbcAMP. At 12 hours, the cells were isolated by centrifugation (435 g for 5 minutes) and recultured in fresh medium in the Mishell-Dutton system. At 60 hours, the cultures were brought to 7.7 µM ^{3}H-BrUdR (specific activity approximately 75 cpm / pmole). Cultures were harvested at the times indicated and exposed to the DNA density analysis procedures of sections III.2.2 and III.2.3. The cesium chloride gradient profile of each time sample was divided into fully density labeled (H/H) ^{3}H-DNA or hybrid density (H/L) ^{3}H-DNA. The ratio of H/H density ^{3}H-DNA to total ^{3}H-DNA x 100 represents the per centage of the cells synthesizing DNA which complete a cell cycle during the time of ^{3}H-BrUdR exposure.


H/H Density ³H cpm / Total ³H cpm x 100
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Hours of ³H-BrUdR Exposure

involving a continuous ^{3}H-BrUdR labeling pattern in combination with the carbonyl iron, Thy-1 serum and complement treatments of the spleen cell population described in section IV.2.1.6 was also attempted.

In spite of these attempts, the cycling time of SRBC-specific spleen cells has yet to be established. The numerous attempts at equilibrium density analysis of SRBC-specific spleen cell DNA failed to yield significant results. Often there was insufficient acid insoluble ^{3}H-DNA cpm in the gradients to determine the gradient profile. Incorporation of both high specific activity ^{3}H-TdR, which was required to yield sufficient label incorporation to allow equilibrium density DNA analysis, and BrUdR inhibited plaque formation such that the apparent doubling time of SRBC-specific cells was much greater than 7 hours. When ^{32}P was substituted for ^{3}H-TdR, equilibrium density analysis of the resulting DNA yielded a background level of ^{32}P which made gradient interpretation impossible.

IV.3.2 The murine myeloma cell system

IV.3.2.1 Growth properties The viability of myeloma cultures was always greater than 90% even in the higher cell concentrations (greater than 1.5 x 10^{6} cells / ml), however, cells appeared very granular and exhibited significant cell surface irregularities at concentrations above 5 x 10^{5} cells / ml. It was found that dilution of the cells to concentrations below 1 x 10^{5} cells / ml retarded their ability to return to exponential growth phase. The upper plateau of the myeloma growth curve (2.5 - 3 x 10^{6} viable cells / ml) does not appear to be a function of cellular nutrition since addition of the nutritional cocktail described in

section IV.2.1.1 did not raise the maximal cell concentration. These observations may be taken as evidence of medium conditioning by myeloma cells influencing their own proliferation.

IV.3.2.2 Myeloma cell cycle kinetics A myeloma cell culture, growing in log phase, was diluted to 2 x 10^5 cells / ml and allowed to grow to 7 x 10^5 cells / ml. Cells were isolated by centrifugation, then recultured at 2 x 10^6 cells / ml in the original conditioned medium with ^{14}C-TdR. After 1 hour, the cells were recultured in another lot of the original conditioned medium at 4 x 10^5 cells / ml. Cultures were harvested from 7 to 17 hours after addition of ^{14}C-TdR. Each culture was labeled with BrUdR for 2 hours prior to harvest. Each culture was exposed to the DNA isolation and density analysis procedures of sections III.2.2 and III.2.3.

Duplicate viable cell estimations of triplicate samples of selected cultures were performed after the 2 hours of BrUdR labeling. Control viable cell estimations were also performed on parallel cultures which either were not exposed to BrUdR or experienced continual BrUdR labeling from the time of removal of the ^{14}C-TdR label from culture. The results of figure 20 indicate that myeloma incorporation of BrUdR for 2 or more hours had little effect on its apparent doubling time. The apparent doubling time of all the cultures was 14 hours.

The results of myeloma cell cycle analysis by the BrUdR technique appears in table 9. Integration of the data of table 9 yields the fraction of incorporated ^{14}C-TdR which is shifted to the hybrid density position at each time point during the analysis of myeloma cell cycling. This data appears in figure 21. The mean cell cycling

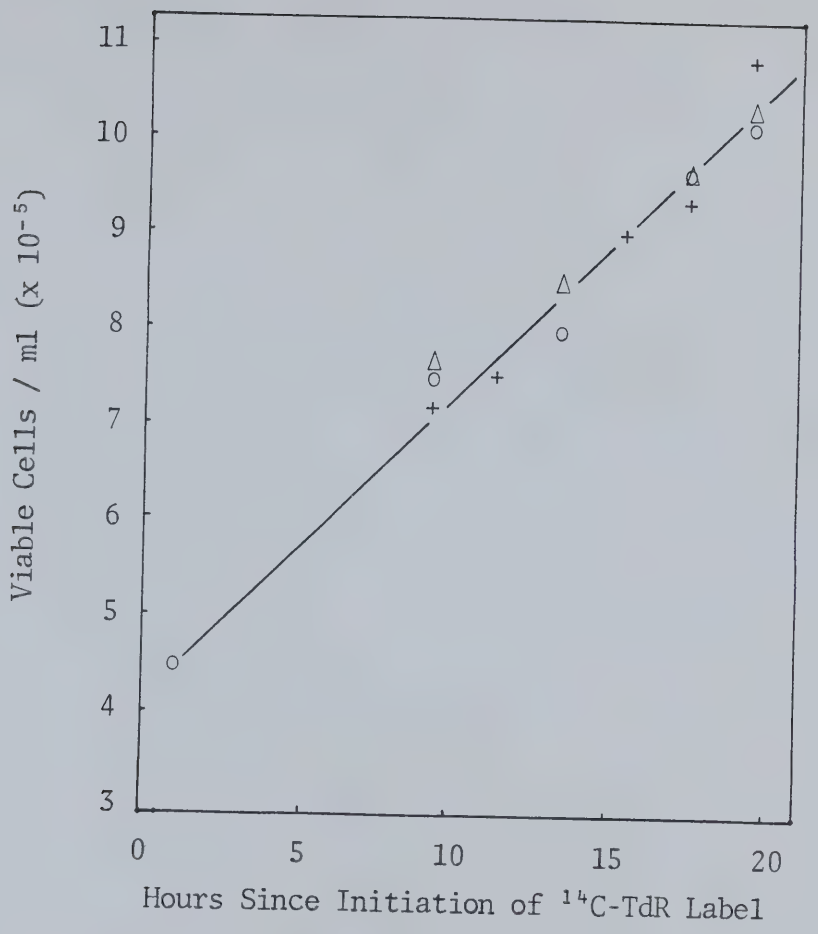


Figure 20. Myeloma doubling time from direct cell counts.

Myeloma cells were grown to 7 x 10^5 cells / ml. Cells were isolated by centrifugation (50 g for 10 minutes) then cultured at 2 x 10^6 cells / ml in the original conditioned medium with 11.5 µM ^{14}C-TdR. After one hour, the cells were isolated by centrifugation (50 g for 10 minutes) and recultured at 4 x 10^5 cells / ml in a fresh lot of the original conditioned culture medium. Some cultures were brought to 11.8 µM BrUdR one hour after ^{14}C-TdR addition (Δ). Other cultures were brought to 11.8 µM BrUdR two hours before harvest at the times indicated (o). Control cultures did not receive BrUdR (+). At the times indicated, triplicate cultures under each labeling condition were analysed for viable cells per ml.

Myeloma cells were grown to 7 x 10^5 cells / ml. Cells were isolated by centrifugation (50 g for 10 minutes) then cultured at 2 x 10^6 cells / ml in the original culture medium with 11.5 µM ^{14}C-TdR. After one hour, the cells were isolated by centrifugation (50 g for 10 minutes) and recultured at 4 x 10^5 cells / ml in a fresh lot of the original culture medium. Cultures were brought to 11.8 µM BrUdR two hours before harvest. Harvested cultures were exposed to the DNA density analysis procedures of section III.2.3. The cesium chloride gradient profile of each time point was defined in terms of hybrid (H/L) and low (L/L) density acid insoluble ^{14}C-DNA. The per centage of the total acid insoluble ^{14}C-DNA cpm found at the hybrid density position represents the fraction of cells synthesizing DNA during ^{14}C-TdR labeling which complete one cell cycle during each BrUdR labeling period. The data in parentheses is the per centage corrected for those cells which have cycled twice since the ^{14}C-TdR labeling.

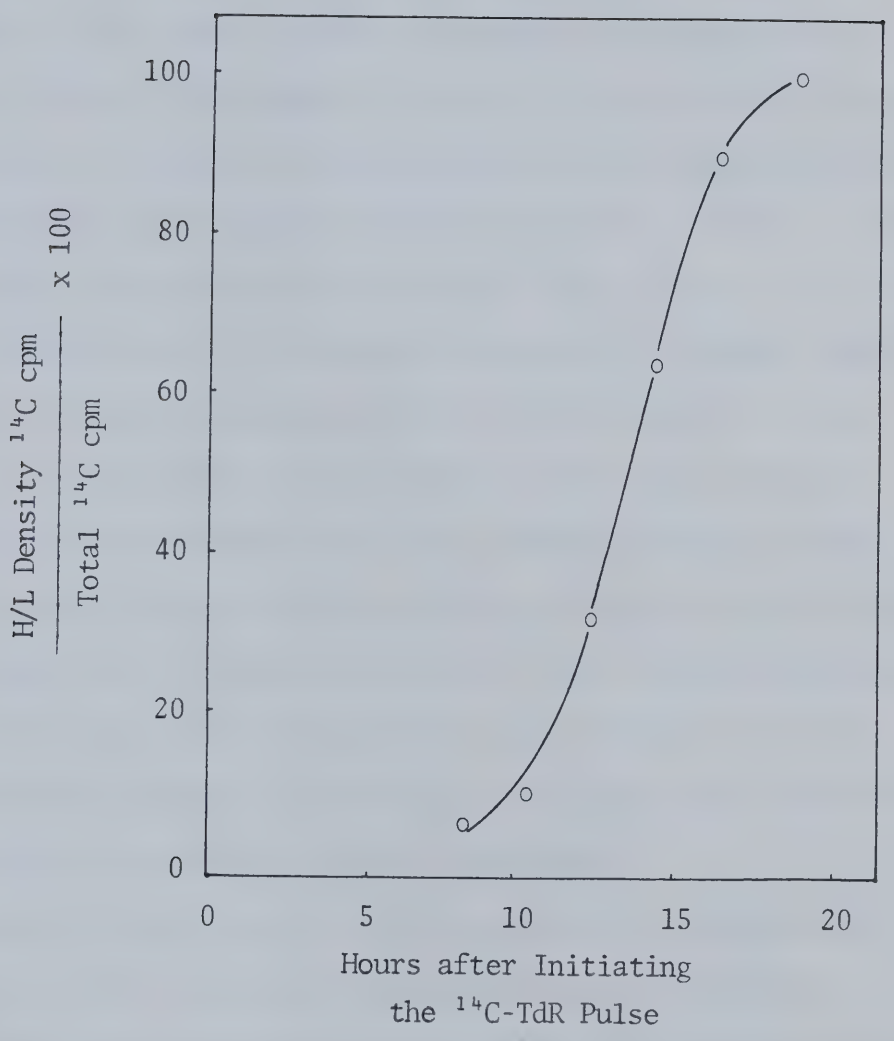
Table 9.

Myeloma Cell Cycling

BrUdR Labeling Time (Hours after ^{14}C-TdR addition)	Per centage of the Total Acid Insoluble ^{14}C-DNA cpm found at the Hybrid Density Position
7 - 9	5.3
9 - 11	5.9
11 - 13	28.6
13 - 15	33.3
15 - 17	29.2 (23.9)
17 - 19	11.2 (5.6)
	102.6

Figure 21. 5-Bromo-2'-deoxyuridine analysis of Myeloma cell cycling.

Myeloma cells were grown to 7 x 10^5 cells / ml. Cells were isolated by centrifugation (50 g for 10 minutes) then cultured at 2 x 10^6 cells / ml in the original culture medium with 11.5 µM ^{14}C-TdR. After one hour, the cells were isolated by centrifugation (50 g for 10 minutes) and recultured at 4 x 10^5 cells / ml in a fresh lot of the original culture medium. Cultures were brought to 11.8 µM BrUdR two hours before harvest at the times indicated. Harvested cultures were exposed to the DNA density analysis procedures of section III.2.3. The cesium chloride gradient profile of each time point was defined in terms of hybrid (H/L) and low (L/L) density acid insoluble ^{14}C-DNA. The ratio of hybrid density ^{14}C-DNA to total ^{14}C-DNA x 100 represents the per centage of the cells synthesizing DNA which pass through one cell cycle during the time since ^{14}C-TdR labeling.


H/L Density ^{14}C cpm / Total ^{14}C cpm x 100
100
80
60
40
20
0
0
5
10
15
20
Hours after Initiating
the ^{14}C-TdR Pulse

time from this data is 14.3 hours with a growth fraction of 102%. The apparent cycling time heterogeneity is from 8 to 18 hours.

IV.4 Discussion

It has become evident that direct application of the BrUdR technique of cell cycle analysis is incapable of answering the question of whether SRBC-specific spleen cells cycle with a mean cell cycling time of 7 hours as the plaque forming cell numbers indicate. At the height of the response to SRBC there are $1 - 5 \times 10^4$ proliferating spleen cells per 10^6 viable cells but only $1 - 5 \times 10^3$ plaque-forming cells per 10^6 viable spleen cells. Therefore, the vast majority of the proliferating cells are not SRBC-specific plaque-forming cells. The cycling time of SRBC-specific cells could be established by selecting for such cells, thereby making the SRBC-specific cells cycling time the major contributor to the mean cell cycling time found by the BrUdR technique. Such selection involves either the isolation of SRBC-specific plaque-forming cells or a reduction of the number of nonSRBC-specific spleen cells after BrUdR labeling but before analysis of the labeled DNA.

Attempts at reduction of nonSRBC-specific cell numbers with carbonyl iron, Thy-1 serum and complement treatments proved unsuccessful. The reduction of viable cell numbers resulting from application of these techniques included a reduction in the number of functional plaque-forming cells. This involved either the death and removal of plaque-forming cells or simply the inactivation of antibody synthesis and secretion. The isolation of rosettes and viable cells through Lymphoprep centrifugation yielded the best selection for SRBC-specific cells, but this technique afforded only

a 5 - 6 fold increase over controls. The most straight-forward means of selection for plaque-forming cells involved micromanipulation of plaque-forming cells from the agarose plaque assay system. However, only a limited number of cells could be isolated from each spleen cell culture (in the order of 200 - 500 cells). This limitation required maximization of the incorporation of thymidine analogues. Such maximization exposed the major limitations of the BrUdR technique of cell cycle analysis, namely, that the incorporation of BrUdR and high specific activity ^{3}H-TdR into spleen cells inhibits their plaque-forming capabilities. This phenomenon of BrUdR inhibition of differentiated cell function has been documented in the spleen cell system (Dutton et al., 1960) as well as in several other cell systems (Walther et al., 1974; Pettengill & Sorenson, 1974). Incorporation of high specific activity tritiated thymidine was required to yield DNA containing sufficient radioactivity to clearly define the DNA density profile. Substitution of ^{3}H-TdR by ^{32}P eliminated the radiation inhibition but resulting cesium chloride gradients were not suitable for kinetic analysis. The BrUdR technique of cell cycle analysis does have definite limitations especially in differentiated cell systems, therefore, sufficient controls must be performed to assess the feasibility of the application of this technique to cell populations.

The cell cycling analysis of whole spleen cell cultures yielded a mean cell cycling time of 13 hours. The apparent doubling time of SRBC-specific cells was 6 - 7 hours based on the increase of plaque-forming cell numbers. This may be taken as an indication that the actual SRBC-specific spleen cell cycling time is 13 hours and that

SRBC-specific cell recruitment may be involved in the increase in plaque-forming cell numbers. Unfortunately, there remains the possibility that the true SRBC-specific cell cycling time was being masked by the majority of the nonSRBC-specific spleen cell cycling.

Application of the BrUdR technique of cell cycle analysis to the murine myeloma cell system proved more successful. The use of ^{14}C-TdR for the initial pulse label did not effect the proliferation of myeloma cells. Myeloma cells readily incorporated BrUdR and there was no inhibition of myeloma cell cycling by up to 18 hours of BrUdR incorporation.

It was important to establish whether the BrUdR technique of cell cycle analysis would yield cell cycle kinetic data comparable to the results of other techniques. The correlation of myeloma cycling as measured by the BrUdR technique (14.3 hours) and direct cell counts (14 hours) was excellent and the growth fraction was found to be approximately 100%. In addition, the BrUdR technique established that myeloma cells exhibit a significant degree of cell cycle time heterogeneity.

In summary, the major points of this thesis are as follows: The analysis of BrUdR labeled DNA as a monitor of cell cycle kinetics is subject to several important limitations. The first limitation is the inability of the technique to establish whether a cell has passed through one or more than one S-phase during the time of analysis. This limits a quantitative analysis of cell cycling to time points at which the data can only be a result of one cell cycle. The second

limitation is the possible inhibition of differentiated function of cells by the incorporation of BrUdR or ^{3}H-TdR. This inhibition can be minimized by employing short pulses of BrUdR, substituting ^{14}C-TdR for ^{3}H-TdR and establishing whether the nucleosides alter the proliferative properties being studied.

The BrUdR technique of cell cycle analysis did , however, give some very important information concerning the cell systems studied. In the myeloma cell system, the BrUdR technique did correlate well with the direct cell count method of monitoring cell proliferation. The BrUdR technique also established the mean cell cycling time of whole SRBC-stimulated, dbcAMP-treated spleen cell cultures to be 13 hours. The failure of the technique to establish the mean cell cycling time of SRBC-specific spleen cells was mainly the result of an inability to isolate sufficient numbers of SRBC-specific cells.

In the Con A stimulated thymocyte system, incorporation of BrUdR had no effect on the areas of thymocyte metabolism tested. Analysis of BrUdR-labeled DNA permitted definition of the absolute thymidine incorporation rate of thymocyte cultures. The BrUdR technique also established the passage of thymocytes through more than one cell cycle as a result of Con A stimulation. This thymocyte cycling was found to be rather heterogeneous in terms of cycling times but the mean cell cycling time was 12.5 ± 1.5 hours with a growth fraction of 0.67. Finally, it was established that thymocytes, once stimulated by Con A, can cycle and respond to Con A for the first time after Con A removal.

The following conclusions can be drawn concerning the general application of the BrUdR technique of cell cycle analysis. The BrUdR technique can easily be used to demonstrate cell cycling. It is also

very useful in establishing absolute incorporation rates of exogenous thymidine. The BrUdR technique can be used for quantitative cell cycle analysis if the absence of deleterious effects of BrUdR is first established. The BrUdR technique, although it is not the complete answer to the technical problems associated with cell cycle analysis, nevertheless, can add considerable information when it is used in conjunction with other kinetic analysis techniques.

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APPENDIX

Publications Related to this Thesis

1. Quantitative analysis of the proliferative activity induced in murine thymocytes by Concanavalin A.

2. Proliferation of murine thymic lymphocytes *in vitro* is mediated by the Concanavalin A-induced release of a lymphokine (costimulator).

3. The role of macrophages in thymocyte mitogenesis.

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QUANTITATIVE ANALYSIS OF THE PROLIFERATIVE ACTIVITY INDUCED IN MURINE THYMOCYTES BY CONCANAVALIN A[1]

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A quantitative analysis of the proliferative response induced in murine thymocytes by concanavalin A (Con A) is described. Exogenous ^{3}H-thymidine labels 35 to 40% of the newly incorporated TMP residues under optimal conditions. The density label 5-bromo-2′-deoxuridine (BrUdR) does not affect DNA metabolism in this system. With this nucleoside, it is shown that newly synthesized DNA is the result of semi-conservative replication, not repair. Double labeling of DNA provides a monitor for cells traversing the cell cycle (S phase to subsequent S phase). The average cycle time is 12.5 hr, and the shortest cell cycle time is 10 hr. The growing fraction of active cells is about two-thirds. The data show that different subpopulations of thymocytes begin proliferating after various times in culture. Once effectively stimulated by Con A, some of the cells can traverse the cell cycle at least twice more after the mitogen is removed.

Lymphocytes differentiate and proliferate in the thymus, and the progeny cells are lost by death and emigration. Thymocyte proliferation is normally controlled by parathyroid hormone and calcium metabolism (1, 2). Differentiation of lymphocytes to mature T cells is controlled by thymic factors, probably acting peripherally and within the thymus (3). Proliferation is observed primarily in the thymic cortex from where the progeny cells move to the medulla before being lost (4). The fraction of lymphocytes proliferating in mouse thymus (approximately 15%) includes lymphoblasts and prolymphocytes (5).

Thymocytes also respond to differentiative and proliferative signals *in vitro*. Various agents, including the thymic hormone(s) and cAMP-enhancing agents, induce differentiative changes in membrane markers (6). Thymocytes proliferate, to a limited degree, in response to T lymphocyte mitogens such as concanavalin A (Con A).

A growing number of investigators have shown that lymphocyte proliferation *in vitro* is profoundly influenced by both soluble, cell-derived factors, and by adherent cells of the macrophage-monocyte series (7–10). To understand these complex interactions more precisely, we have developed quantitative methods for analyzing the proliferative response of mouse thymocytes to Con A. Using this approach, we have shown that proliferation requires both the lectin and a cell-elaborated "costimulator". The elaboration of costimulator requires lectin, T cells, and an adequate number of macrophages (11, 12).

In this paper, we describe how the incorporation of ^{3}H-thymidine under defined conditions can be related to the absolute level of DNA synthesis. A technique, adapted from the work of Munakata and Strauss (13), is described for determining the cell cycle time and growth fraction of these cells. It requires a double label of cellular DNA, using as one label BrUdR[5] to shift the DNA density. DNA labeled with a radioactive isotope at one particular time becomes hybrid in density when it replicates subsequently with the density label present (Fig. 1). If the nucleoside can be shown to have no effect on the response being studied, this method is an attractive one, compared to the techniques utilizing cell arrest by thymidine block or colchicine metaphase arrest. The density label technique also determines whether mitogen-stimulated cells from which the mitogen has been removed can subsequently traverse the cell cycle. In this paper, the use of BrUdR is first shown to have no detectable effect on the parameters being studied, except for density labeling the DNA. The technique is used to determine the cell cycle time for these cells, and the heterogeneity of the response in the thymocyte population.

MATERIALS AND METHODS

Tissue culture medium and fetal calf serum were from Grand Island Biologicals Co., Grand Island, N. Y. ^{3}H-BrUdR, ^{3}H-TdR, and Omnifluor from New England Nuclear, Boston, Mass., MAG, Con A and Pronase (B grade) from Calbiochem Corp, La Jolla, Calif., HEPES from Sigma Chemical Co., St. Louis, Mo., glass fiber filters (GF/C 2.5 cm) from Whatman Co., Clifton, N. J., CsCl from Pierce Chemical Co., Sarkosyl from Ciba-Geigy, Ardsley, N. Y., and BrUdR from PL Biochemicals, Milwaukee, Wis.

Cells and culture conditions. CBA/J Mice were purchased from Jackson Laboratories, Bar Harbor, Maine, and bred and maintained at the University of Alberta facilities. Mice were killed at 5 to 8 weeks of age and the thymus tissue was removed. The tissue was minced and passed through a fine stainless steel screen. Cells were collected by centrifugation at $70 \times G \times 7$ min. These conditions left the smallest lymphocytes, about 30% of the total cells, in the supernatant. The small cells, which respond only poorly to mitogenic stimulation, were discarded. Cells were cultured in minimal Eagle's medium containing 36 mM sodium bicarbonate, 10 mM HEPES buffer, 0.43 mM sodium pyruvate, 8.5% fetal calf serum and 1×10^{-5}

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[5] Abbreviations used in this paper: TdR, deoxythymidine; MAG, methyl-α-glucopyranoside; BrUdR, 5-bromo-2′-deoxyuridine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

M 2-mercaptoethanol. Unless otherwise indicated, cultures were set up in 16 x 100-mm glass test tubes. They were incubated at 37°C in 10% CO_2 in air at 100% relative humidity. Viable cells were counted by eosin Y exclusion.

Measurement of thymidine uptake. The optimal conditions for labeling DNA with ^{3}H-TdR were determined as described in the *Results*. The standard conditions were to add 10 μM ^{3}H-TdR of specific radioactivity giving 10 to 40 cpm/pmole under the counting conditions used. After 3 to 6 hr, cells were harvested by filtration through GF/C filters and washed with cold 0.85% NaCl, followed by cold 5% trichloroacetic acid, and then ethanol. The dried filters were counted in toluene-Omnifluor. To facilitate comparisons, most data are expressed as pmole ^{3}H-TdR incorporated per hour per 10^6 cells, as described in the *Results*. Values given are the averages of triplicate cultures. The average standard deviation was 11%.

Isolation of DNA. Cells were harvested by centrifugation for 360 × G × 7 min, washed, and resuspended in 0.5 ml 0.85% NaCl. To the suspension was added: 0.1 ml 50 mM Tris (Cl), pH 7.5, 10 mM EDTA; 0.2 ml 5% Sarkosyl, and 0.2 ml at 10 mg/ml pronase. After incubation at 40°C for 4 hr, the mixture was extracted twice with phenol- 0.1% 8-hydroxyquinoline, twice with chloroform-isoamyl alcohol (24:1), and twice with ether. Density gradient analysis was performed on samples made up with CsCl to the indicated starting density. Centrifugation was for 48 hr in a Beckman Ti50 rotor at 40,000 rpm and 15°C. Gradients were pumped out and fractionated directly onto 3MM filter paper discs. The discs were washed with 5% trichloroacetic acid and ethanol and counted in toluene-Omnifluor. Isotope overlaps were corrected where necessary. The densities of selected fractions were determined refractometrically.

Density labeling of replicating DNA. This was achieved by adding 3 to 10 μM BrUdR to cultures. In several experiments, radioactive and density labels were introduced together by adding both BrUdR (4 μM) and ^{3}H-TdR (0.02 to 0.1 μM). The tritium then appears in the density-labeled DNA; this low level of TdR does not interfere with the uptake of BrUdR, as determined by the resulting density of the DNA. In some experiments we have used ^{3}H-BrUdR. No other agent was added to enhance BrUdR uptake. From the observed density shifts, 25 to 30 mg/cm^3 for hybrid density DNA, about 50 to 60% of the thymidine residues were being substituted with BrUdR (14).

RESULTS

Thymidine uptake as a relative measure of thymocyte proliferation. The uptake of ^{3}H-TdR into acid-insoluble material (thymidine uptake) was to be used as a measure of thymocyte proliferation in this work. It was first necessary to show that differences in thymidine uptake represented differences in proliferation and not, for example, differences in the thymidine pools of the culture. A representative saturation curve for thymidine uptake by thymocytes stimulated for 48 hr with Con A is given in Figure 2. (The time course of response is fully described in reference 11.) The system exhibits simple saturation kinetics in the range shown, giving a linear double reciprocal plot and yielding an apparent endogenous thymidine level of 0.6 μM. Experiments were routinely performed with 10 μM thymidine (90 to 95% saturation).

The uptake of ^{3}H-BrUdR, when plotted in double reciprocal form, also fell on straight line (data not shown), with half-saturation at 0.66 μM. At saturation, BrUdR incorporation (pmole/hr/10^6 cells) was 1.5 times as high as TdR uptake. The two nucleosides showed the same time course of uptake (data not shown).

The rate of TdR uptake was constant up to at least 12 hr of labeling (Fig. 3). The apparent falling off from the expected accumulation is at least partly due to the turnover of incorpo-

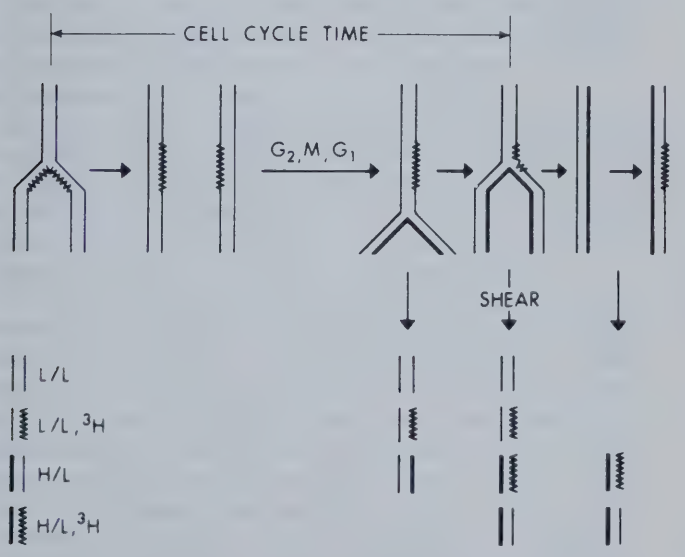

Figure 1. Determination of the cell cycle time by double labeling of DNA. A pulse label of ^{3}H-TdR is incorporated into DNA that is undergoing replication. After the pulse, the ^{3}H DNA is of fully light (L/L) density. In the presence of BrUdR, it becomes hybrid (H/L) in density exactly one cell cycle time after administration of the ^{3}H pulse. Shearing normally accompanies isolation of the DNA, and the resulting pieces are short relative to the length of DNA replicated during the ^{3}H pulse (1 to 2 hr). The ^{3}H label in the H/L fragments bands at fully light density after denaturation.

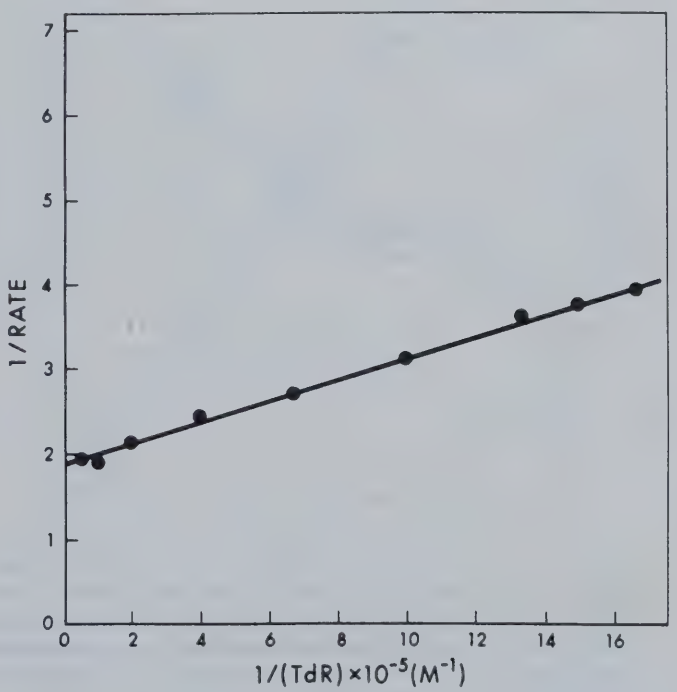

Figure 2. Saturation kinetics for ^{3}H-TdR. Cultures were incubated for 48 hr in 4.5 μg/ml of Con A, and labeled with various concentrations of ^{3}H-TdR for 4 hr. The line is least squares-determined. Rate of thymidine uptake is in pmole/hr/10^4 cells.

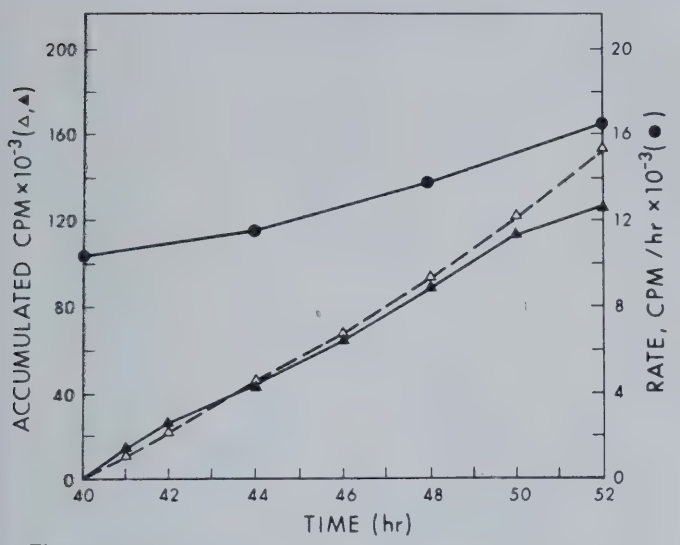

Figure 3. Linearity of thymidine uptake with time. Cells were cultured in 4.5 μg/ml Con A for 40 hr. Cultures were labeled with 10 μM ^{3}H-TdR, either for 2 to 3 hour pulses to determine the instantaneous rate (●), or continuously (▲). The expected cumulative uptake (△) is determined from the integrated rate curve.

rated label, determined to have a $t_{½}$ of 48 hr (data not shown). Experiments were routinely performed with 3- to 6-hr-labeling times.

BrUdR does not alter replication or growth. Since BrUdR has a number of effects on various biologic systems (15, 16) it was important to determine if it affected growth or DNA synthesis and degradation in Con A-stimulated thymocytes. In one set of experiments, DNA was labeled with ^{14}C-TdR for the first 24 hr. The loss of acid-precipitable ^{14}C label, reflecting cell death and DNA degradation, was the same with or without 4 μM BrUdR present (Fig. 4). The incorporation of ^{32}P-orthophosphate into acid-insoluble material was similarly unaffected by BrUdR (Fig. 4). The ^{32}*P*-labeled material was isolated at 70 hr of culture and deproteinized. Of the ^{32}*P*-labeled nucleic acid (41% of the total), 75% was solubilized by pancreatic DNase in both samples (± BrUdR present). Thus, in this system BrUdR has no demonstrable effect on DNA metabolism.

Extensive BrUdR incorporation also had no significant effect on cell viability. Con A-stimulated cultures incubated with 4 μM BrUdR from 20 hr contained (per ml) 0.36×10^6 viable cells at 48 hr, compared to 0.35×10^6 for controls. Having BrUdR present from 35 to 45 hr did not affect the viable cell count at 75 hr (0.40×10^6 *vs* 0.37×10^6 for controls).

BrUdR is incorporated during replication. Essentially all of the BrUdR incorporated was by semi-conservative DNA synthesis. This was shown by labeling DNA first with ^{14}C-TdR (between 40 and 50 hr of culture) and then with tritium and BrUdR (55 and 65 hours). Under these conditions (total elapsed labeling time 25 hr) no cells passed through S phase three times, but a large fraction did go through S phase twice, first being ^{14}C labeled, the next time being converted to ^{3}H-^{14}C hybrid density DNA (Fig. 5*a*). The density of this hybrid was 29 mg/cm^3 greater than that of normal DNA (average of three experiments). The same DNA sample after heat denaturation separated into fully low density ^{14}C and fully high density ^{3}H, as expected for semiconservative synthesis (Fig. 5*b*). Repair synthesis would be reflected by ^{3}H label in low density chains, which in fact did not exceed 5% of the total in three experiments. Re-utilization of ^{14}C via breakdown and reincorporation into high density chains also did not occur.

Measurement of cell cycle time and growth fraction. The cell cycle time defined in Figure 1 is the time between replications of the same DNA sequence in parental and daughter cells. Con A-stimulated cells were labeled with ^{3}H-TdR between 35 and 36 hr of culture. The ^{3}H-TdR was removed and the cells were cultured further in Con A. At 40 hr, 4.4 μM BrUdR was added, and cultures were harvested at the time s indicated in Figure 6. DNA labeled with ^{14}C was added as an internal recovery and density marker. The tritium pulse-labeled DNA was completely light in density 7 hr after initiation of the ^{3}H labeling. There was a barely detectable amount (1.8%) of hybrid density DNA by 9 hr, and progressively more after that. About 75% of the surviving tritium-labeled DNA eventually became density labeled by 24 hr. Half of this amount was shifted at 12.5 hr. Of the tritium-labeled DNA present at 7 hr, 67% eventually recycled at least once. From Figure 6 it is evident that cell death and loss of DNA affected the cycling cells to the same extent as non-cycling cells.

Kinetics of thymocyte cycling. Progeny of cells which are active in the first, mitogen-independent, phase of DNA synthesis (0 to 24 hr, see Reference 11) were no longer proliferating by 55 hr, as shown by the data in Table I. Addition of a density label at this latter time caused no density shift of ^{14}C label incorporated into DNA between 0 and 25 hr. Of the progeny of cells labeled about two generation times before BrdU labeling (that is, between 25 and 40 hr of culture), 43% were still active, and, as already seen in Figure 6, about 62% of the immediately preceding generations' progeny cycle through S phase. The data are consistent with a growth fraction at this time in culture, of about two-thirds.

Another examination of thymocyte cycling is provided by Table II. In this case BrUdR was continuously present from 15

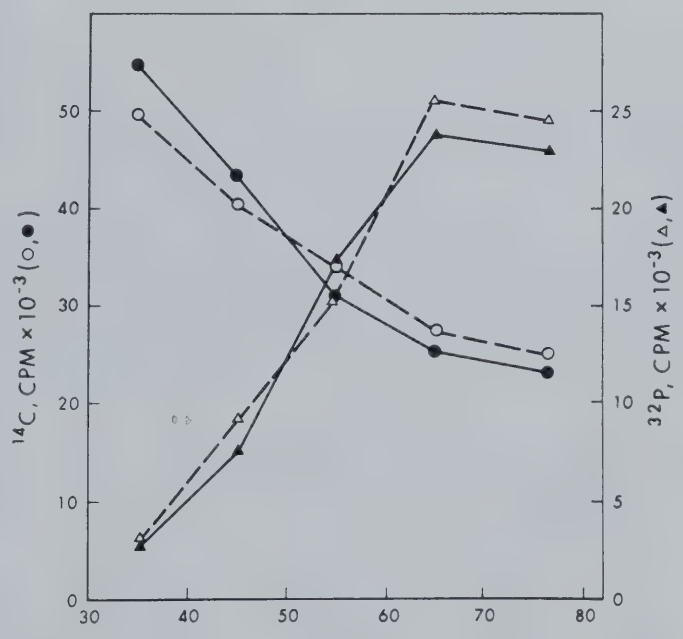

Figure 4. BrUdR does not affect DNA metabolism in Con A-stimulated thymocytes. Cells were cultured (3×10^6/ml) with Con A. One group of samples was labeled with ^{14}C-TdR from 0 to 24 hours, and the disappearance of acid-precipitable radioactivity was monitored either in the absence (○), or presence (●), of 4 μM BrUdR, that was added at 24 hr. Another group of cultures was labeled with 0.5 μCi/ml ^{32}Pi from 24 hr, either in the absence (△), or presence (▲), of 4 μM BrUdR. Approximately 31% of the acid-insoluble ^{32}P-labeled material was DNA (see text).

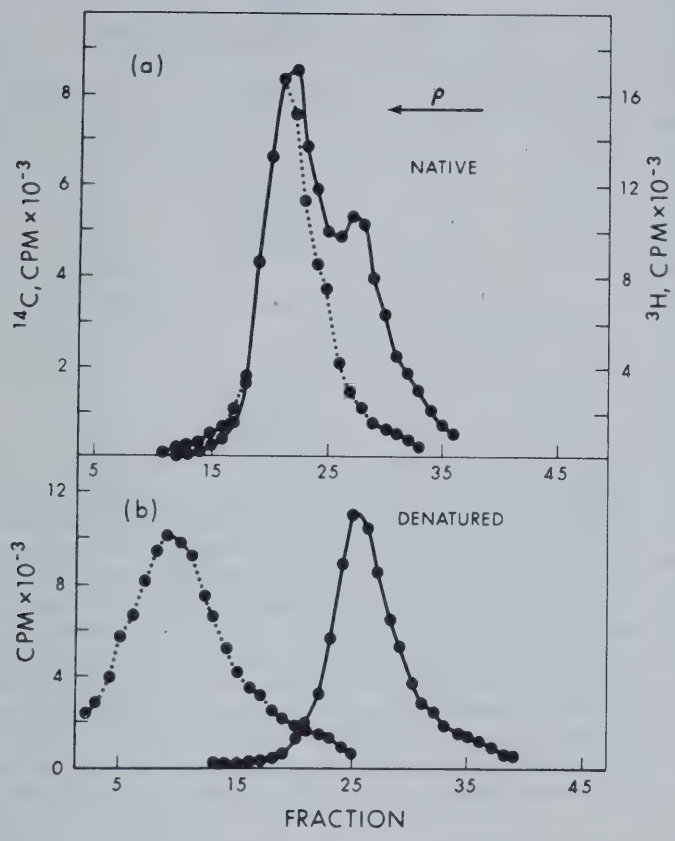

Figure 5. BrUdR is incorporated into replicating DNA. Thymocytes were cultured with Con A for a total of 65 hr. DNA was labeled with ^{14}C-TdR from 40 to 50 hr, and with 4 μM BrUdR-0.021 μM ^{3}H-TdR from 55 to 65 hr. The native isolated DNA (see *Methods*) was centrifuged at 40,000 rpm and 5°C for 60 hr, in CsCl of initial density 1.75 g/ml (*a*). The ^{14}C peak to the right corresponds in density to fully light DNA (—●—); the larger peak of ^{14}C and ^{3}H is 25 to 30 mg/cm^{3} higher in density (H/L) (---●---). The heat-denatured sample shown in *b* demonstrates that BrUdR is incorporated during replication, not repair; the fully heavy ^{3}H peak is about 70 mg/cm^{3} higher in density than the fully light ^{14}C peak.

hr; tritium label was added at 55 hr and all cultures were harvested at 56 hr. The data show that some cells went through at least two cell cycles after Con A was removed. Thus, when Con A was removed by washing cells with MAG (13, 17) at 25 hr, about 15% as many cells were active by 56 hr as when Con A was present throughout (line 4 *vs* line 1). About two-thirds of the ^{3}H label went into heavy/heavy DNA. Since no cells active before 25 hr are active by 56 hr (Table I), the full density shift, requiring two traverses of S phase, must have occurred after the mitogen was removed. In other words, some cells were committed to at least two further cell cycles after 25 hr exposure to Con A, and these cycles occurred even if the mitogen was removed.

The striking feature of the data in Table II is that in each case about one-third of the newly synthesized DNA was of hybrid density, that is, one chain out of six was derived from the ancestor cell taken from the thymus. This ratio (1:3 heavy to light in double-stranded DNA) is achieved between two and three generations in BrUdR medium (see *Discussion*).

DISCUSSION

The kinetics of DNA synthesis and degradation, and cell growth and viability were unaffected by BrUdR under the conditions used here. Sufficient BrUdR was incorporated without having to add FUdR (18, 19) to produce 60% of the theoretical density shift (observed shift of hybrid DNA 0.029 g/cm^{3}, theoretical shift for 100% substitution in one strand 0.048 g/cm^{3}; Reference 14).

For ease of comparison, and to obtain an idea of the magnitude of the response, we have used the parameter pmole TdR incorporated/hour/10^{6} cells cultured. If all of the cultured cells were growing, and if all the newly-incorporated TMP residues were derived from the exogenous, radioactive TdR, the incorporation rate would be about 470 pmole/hr/10^{6} cells. This is derived from the DNA content of mouse cells, about 6.5 pg (20), a cell cycle time of 12.5 hr, as determined here, and a DNA composition of 29% thymine (20). Since the rate of TdR uptake was two-thirds that of BrUdR (see *Results*), and BrUdR incorporation was 60% of theoretical, the expected rate of ^{3}H-TdR uptake if 100% of the cells were active would be 190 pmole/hr/10^{6} cells cultured. This value is, in fact, approached under conditions described in a subsequent paper (12).

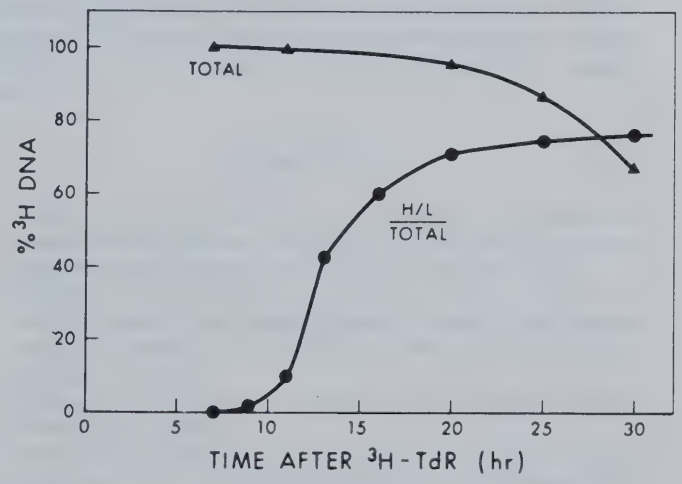

Figure 6. Cell cycle time and growth fraction of Con A-stimulated thymocytes. Con A-stimulated cells were labeled with ^{3}H-TdR from 35 to 36 hr, washed, and re-cultured with Con A. They were continuously exposed to 4.4 μM BrUdR from 40 hr onward. At various times after 35 hr, cultures were harvested and lysed in the presence of ^{14}C-labeled DNA as a recovery marker. The purified DNA was centrifuged to equilibrium in CsCl gradients, and the fraction of hybrid density was determined. Results are corrected to constant recovery of ^{14}C marker in the gradients. The maximal fraction of the original ^{3}H label that appeared as hybrid density DNA was 0.67 at 20 hr (recovery of 0.95 × fraction shifted, 0.7).

TABLE I

Re-cycling of Con A-stimulated thymocytes

^{14}C-TdR label	^{14}C per Gradient	Fraction of ^{14}C in Density Position	
		L/L	H/L
hr	*cpm*		
0–25	9,670	1.0	0
25–40	39,300	0.57	0.43
40–50	77,000	0.38	0.62

Thymocytes were cultured with Con A for 65 hr in each case. DNA replicating during the intervals indicated in column 1 was labeled with ^{14}C-TdR, which was then removed. At 55 hr, 4 μM BrUdR and 0.021 μM^{3} H-TdR were added to introduce a density and tritium label into newly replicated DNA. DNA was isolated at 65 hr and analyzed by CsCl gradient centrifugation (see Fig. 5). DNA replicating between 55 and 65 hr is of H/L (hybrid) density.

TABLE II

The effects of Con A removal on cell cycling

Control Cells Washed at:	^{3}H per Gradient	Fraction of ^{3}H in Density Position	
		H/L	H/H
hr	*cpm*		
25	39,700	0.27	0.73
35	42,400	0.35	0.65
45	39,600	0.31	0.69
Con A removed at			
hr			
25	5,900	0.29	0.71
35	18,200	0.28	0.72
45	32,100	0.33	0.67

Con A stimulated thymocytes were exposed continuously to BrUdR (4 μM) from 15 hr until the end of culture, at 56 hr in each case. Con A was removed from the second set of samples by adding 47 mM MAG and washing at the times indicated. Cells were then re-cultured in Con A-free medium. Control cells were simply removed from their medium and re-cultured in fresh Con A medium. At 55 hr, all cultures were labeled with a tracer amount (0.02 μM) of ^{3}H-TdR. DNA synthesized during the 55 to 65 hr interval thus was both density and tritium labeled. Cells with fully light (unreplicated) DNA would yield only H/L daughter molecules if they traverse S phase at this time. Cells one generation removed (H/L DNA) would yield one daughter each of H/L and H/H DNA. Cells with H/H DNA would of course produce H/H DNA during replication.

The importance of determining the absolute proliferative activity of lymphocyte cultures lies partly in the need for quantitative assays in studying the effects of macrophages and soluble factors. In our studies of such effects in thymocyte system (11, 12), it has become clear that simply reporting a "stimulation index" is often of little value in estimating the strength of a given effect since basal levels of proliferative activity can vary widely. Moreover, it is important to know how much of a given activity is elicited in determining conditions or cells which produce it.

Another objective of this work was to determine cell cycle kinetics without resorting to agents such as colchicine, which can cause artifacts (21, 22), perturbing the cells. Contrary to results obtained with human peripheral lymphocytes, where re-stimulation was necessary to induce cells to go from S phase to subsequent S phase (13), thymocytes complete this cycle in the absence of Con A, once stimulation is effected. From the results of Table I and II, it appears that the effect of leaving Con A in the culture is to bring more and more cells into the response. Some cells are apparently giving an initial response throughout the first 50 hr or so of culture. This is also implicitly evident from the change in DNA synthesis rates (11). The rate of TdR uptake increases by about 6-fold between 24 and 50 hr. With an average cell cycle time of 12.5 hr and a growth fraction of two-thirds, the result of a synchronized turn-on at 24 hr would be only a 1.8-fold increase in numbers of proliferating cells by 50 hr. The difference, it seems necessary to hypothesize, is due to non-synchrony in turn-on time, some cells entering the proliferation cycle much later than others.

Heterogeneity of response has been proposed by Gunther *et al.* (23) for Con A-stimulated human peripheral blood lymphocytes. In that case, all of the cells able to respond appeared to be committed, and thus no longer requiring Con A, by 20 hr. Similar results have been reported for rat lymph node cells (17).

The data and interpretations presented here and in other work (11) suggest that thymocytes include a heterogeneous population of lymphocytes. One fraction (about 10%) actively proliferates at the outset of culturing, and is mitogen independent. These may be the cells active *in vivo* (4, 5). Subsequently, beginning at 24 hr, various subpopulations begin to proliferate and then die. The recruitment requires both a thymus-derived costimulator (11) and the mitogen, at least during the first cycle. Subsequent cycles are mitogen independent. About one-third of the pulse-labeled DNA at 56 hr was of hybrid density when cells had been growing in BrUdR continuously (Table II). One model explaining these data is that, on the average, the active population at 56 hr was passing through S phase for the second or third time (two passages would produce 1:2 H to L DNA, three passages, 1:4).

The cell cycle time of Con A-stimulated thymocytes in culture, about 12 or 13 hr, is greater than the 6.8 to 8.2 hr estimated by Metcalf for thymocytes *in vivo* (24). Antigen-responsive (25) and PHA-stimulated (26) lymphocytes have been shown to have cell cycle times of 13 and 14 hr, respectively.

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PROLIFERATION OF MURINE THYMIC LYMPHOCYTES IN VITRO IS MEDIATED BY THE CONCANAVALIN A-INDUCED RELEASE OF A LYMPHOKINE (COSTIMULATOR)[1]

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Mitogen-induced proliferation of lymphocytes may in theory result directly from the interaction of mitogen with the cells, or indirectly as a result of the mitogen-stimulated release of lymphokines. In the case of murine thymic lymphocytes exposed to concanavalin A (Con A) in tissue culture, we have determined that mitogenesis depends upon a lymphokine. Interaction of the thymic lymphocytes with lectin is necessary, but not sufficient, for mitogenesis. A lymphokine, or costimulator for mitogenesis, is released by normal spleen or thymus cells during the first 16 hr of their exposure to Con A, and in the presence of a phytomitogen it stimulates thymic mitogenesis. Under conditions of low costimulator levels, no mitogenesis follows the interaction of Con A with cells. The response of adult CBA/J mouse thymocytes to phytohemmaglutinin (PHA) is very low, compared to their response to Con A. When costimulator is added to PHA, the cells respond as well as they do to Con A. Costimulator does not act through Con A-binding sites on thymus cells. Its production is dependent on both cells carrying θ surface antigen (T lymphocytes) and adherent cells of the macrophage-monocyte series. The adherent population, but not the T cells, may be heavily irradiated without affecting production of costimulator. Costimulator is not a mitogen on its own.

The proliferation of lymphocytes in response to phytomitogens is often taken as a model for the proliferative aspect of immune responses. This model appears now to be fairly complex. Among its complexities are the interactions between distinguishable type of cells, both synergistic and antagonistic, and the presence of cell-produced lymphokines, which can also be either stimulatory or inhibitory. Stimulatory factors have been reported to arise in a large number of lymphocyte systems. Human peripheral lymphocytes (1, 2) guinea pig lymph node cells (3), and mouse peritoneal exudate cells (4) have been found to produce various stimulatory lymphokines. Both lymphocytes (5) and cells of the macrophage-monocyte series (2, 6, 7) have been implicated as the source of such materials. Many of the reported factors are apparently mitogenic in themselves (2, 3, 5, 7, 8), whereas others potentiate mitogenic agents (4, 9, 10).

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Mouse thymocytes differ from other commonly-used sources of lymphocytes (spleen, lymph node, and blood leukocytes) in containing relatively few B lymphocytes or cells of the macrophage-monocyte series. About 15% of thymic lymphocytes proliferate *in vivo*, and a similar fraction can be stimulated to proliferate by concanavalin A (Con A) *in vitro* (11). The proliferating subpopulation *in vitro* has a growth fraction of about two-thirds and an average cell cycle time of 12.5 hr. (11). After about 72 hr in culture, proliferation stops and quiescence sets in. In a subsequent paper, we describe the requirement for macrophage cells in both the initial response and in reversing the quiescent state (12). In this paper, we describe a cellularly-produced factor, released in response to mitogenic stimulation, which is necessary for thymic lymphocyte mitogenesis. It differs from most lymphokines in being non-mitogenic, but is essential for lectin-induced mitogenesis in this system. For this reason, we refer to it as a costimulator of mitogenesis.

METHODS

Anti-θ ascitic fluid. This was prepared in AKR/J mice by repeated weekly injections of CBA/J thymocytes, as described elsewhere (13). Spleen cells (1.8×10^7 in 1 ml) were treated with 1:5 diluted anti-θ fluid, followed by agarose-absorbed guinea pig complement (13). About 30% of the initial cells were removed by anti-θ plus complement.

Gamma irradiation. This was done in a ^{137}Cs source. The dose was 2500 rads, sufficient to diminish ^{3}H-TdR uptake of the irradiated cells to less than 5% of control values.

Depletion of macrophages, and other materials and methods were as described elsewhere (11, 12).

RESULTS

The effect of decreasing the cell/volume ratio. Thymic lymphocytes, cultured with Con A, respond by synthesizing DNA for a limited time. There is an initial, declining, mitogen-independent phase of DNA synthesis (14) (Fig. 1) followed by a rapid rise in the rate of DNA synthesis. To determine whether "medium conditioning" played an important role in the response, cultures were set up in tubes with 4×10^6 cells each and variable total volumes of medium (Fig. 1). At very low cell to volume ratios there was essentially no stimulation of mitogenesis by Con A. Increasing this ratio had two effects; i) the level of stimulation increased, and, ii) the maximum stimulation occurred earlier. These data suggested that contact with mitogen alone by thymocytes was inadequate for stimulation, and that cells were strongly stimulated only in conditioned medium. This conclusion was further supported by "double chamber" cultures (data not shown), in which cells in small inner tubes immersed in a relatively large volume of

medium (overall cell to volume ratio 0.5×10^6/ml) were only poorly stimulated by mitogen. Adding cells to the outer chamber to increase the overall ratio to 2×10^7/ml caused the cells in the inner tube to respond 2.3 times as well. Thus, a diffusible material other than Con A is important to the mitogenic response of thymocytes ("costimulator").

Demonstration of a soluble costimulator. A direct test of the putative, diffusible costimulator was performed as follows. Cultures at a high cell to volume ratio (8×10^6/ml) were harvested at various times, and the medium was transferred to fresh cell cultures where the ratio was 1.0×10^6/ml. As shown in Figure 2, the high density culture supernatant removed at 16 or 24 hr demonstrated a strong costimulator activity, as assayed on low density cells. When transferred at 72 hr, the high density culture medium was no longer stimulatory. This has important implications for the interpretation of the experiments (see *Discussion*). A similar experiment (data not shown) showed that the costimulator activity was much lower at 6 hr than at 12 hr. The test cells were higher in their response at 66.5 hr than at 41.5 hr when costimulator generated by 16- or 24-hr first-stage cultures was present. The first-stage cultures, on the other hand, had exhausted the costimulator by 48 hr and were quiescent by 72 hr, presumably because at the high cell density costimulator was inactivated more rapidly.

Production of costimulator by thymus and spleen cells. Medium taken from either spleen or thymus cells cultured at high cell to volume ratios with Con A present contained costimulator activity. Con A was essential for costimulator production (Table I). Production, or release, of costimulator was over by 17 hr of primary culture. In subsequent work, spleen cells have been found to produce higher levels of costimulator than thymus cells, as shown by dilution of the medium (work in progress). For the routine production of costimulator (cf., Table III), medium from spleen cells cultures containing from 8 to 20 million cells/ml was used at 3- to 40-fold dilution.

Production of costimulator requires both T lymphocytes and macrophages. A concentrated suspension of normal spleen cells in Con A-containing medium was used to generate costimulator, which was assayed (after 12-fold dilution) on fresh, dilute

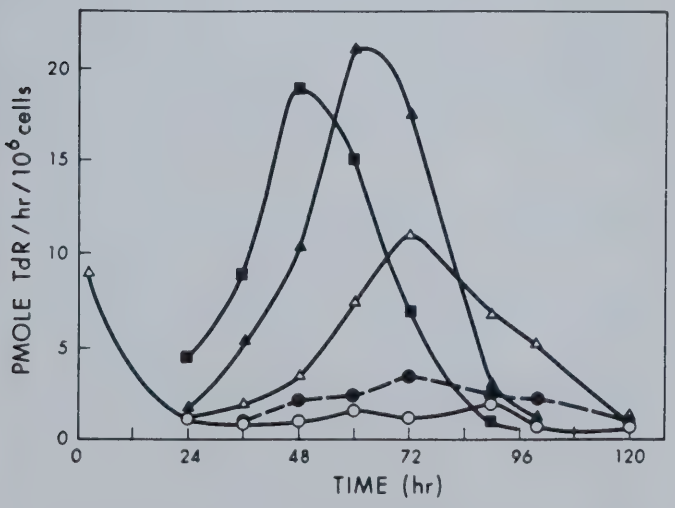

Figure 1. Effect of the cell/volume ratio on thymocyte stimulation by Con A. Thymocytes, 4×10^6 per culture in round-bottomed glass tubes, were cultured with 7.5 μg/ml of Con A, and various volumes of medium. Cell:volume ratios: ○, 0.5×10^6/ml; ●, 1×10^6/ml; △, 2×10^6/ml; ▲, 4×10^6/ml; ■, 8×10^6/ml.

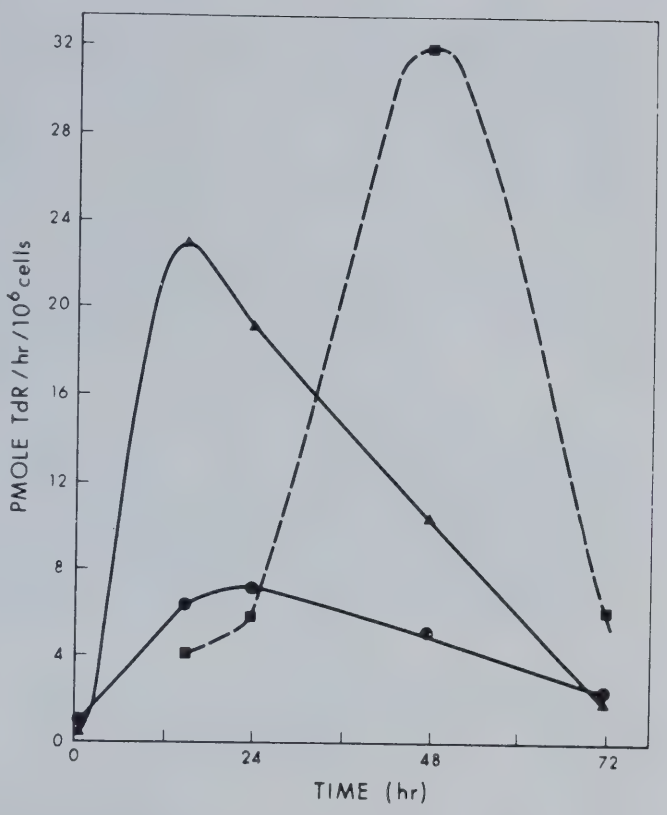

Figure 2. Assay for costimulator activity. Thymocytes were cultured in 6 μg/ml Con A at 8×10^6 cells/ml for the times indicated on the abscissa. The culture supernatants were obtained by centrifugation, and frozen. The thawed media were then used to prepare cultures of fresh thymocytes at 1.0×10^6/ml. The second stage cultures were assayed for thymidine uptake at 41.5 hr (●), and 66.5 hr (▲). The activity of the first-stage or medium-conditioning cultures is shown by ■. Medium containing Con A was also frozen, thawed, and used as a control. The control values are shown at 0 hr on the abscissa.

TABLE I

Production of costimulator

Culture Supernatant from	Activity
17 hr thymocytes + Con A	19.6
17 hr thymocytes − Con A	1.6
17 hr spleen cells + Con A	18.2
17 hr spleen cells − Con A	0.6
17 hr medium + Con A	0.8
17 hr medium − Con A	0.6
30 hr thymocytes + Con A	1.0
30 hr thymocytes − Con A	0.2
30 hr spleen cells + Con A	5.4
30 hr spleen cells − Con A	0.8
30 hr medium + Con A	1.0
30 hr medium − Con A	0.6
Fresh medium + Con A	0.4

Culture supernatants were removed after 17 hr of incubating 8×10^6 cells/ml, as indicated. The cells were resuspended with fresh medium and cultured a further 13 hr to produce the 30-hr supernatants, which thus contain material elaborated by the cell culture between 17 and 30 hr. The culture supernatants were made up to 6 μg Con A/ml as required, and added to fresh thymocytes, at 0.5×10^6/ml, neat. Thymidine uptake rate (pmol/10^6 cells/hr) was measured at 73 hr of these secondary cultures as costimulator activity.

TABLE II

Production of costimulator by irradiated or anti-θ treated spleen cells

Source of Costimulator	Activity
1. None	1.2
2. Normal spleen cells	64
3. Complement-treated spleen cells	69
4. Anti-θ-plus-complement-treated spleen cells	5.0
5. Irradiated spleen cells	8.8

The amount of costimulator produced by various cell populations was determined by adding the conditioned medium, diluted 1:12 with normal medium, to fresh cultures of dilute thymocytes (0.5×10^6/ml). Under these conditions (line 1) thymocytes give a poor response due to lack of costimulator (see Fig. 1). Con A was at 6 μg/ml in the costimulator-generating cultures, and at 3 μg/ml in the thymocyte assay cultures. Anti-θ treatment and irradiation (*Methods*) were done just before initiating the costimulator-generating cultures, which were incubated for 27 hr before harvesting the medium. Each milliliter of costimulator-generating culture contained the surviving cells from 12×10^6 spleen cells. The assay cultures of dilute thymocytes were incubated 72 hr. Activity of the assay cultures was measured as in Table I.

thymocytes. The production of costimulator was reduced by more than 94% after treatment with anti-θ serum plus complement (Table II). Irradiation of the spleen cells immediately before culturing them with Con A reduced the production by more than 90%. The apparent inhibitory effects of anti-θ serum and irradiation are likely to be underestimates, since at the level of costimulator produced, the system (fresh thymocytes) gave a very strong response, and was probably saturated with costimulator. At 65 pmole/hr/10^6 cells, 34% of the thymocytes are responding (11), which is higher than seen in untreated, optimal, cultures.

A direct synergistic effect between radiation-resistant spleen cells, presumably Mφ, and Mφ-depleted thymocytes, is shown in Table III. Medium taken from either normal thymocytes, Mφ-depleted thymocytes, or heavily irradiated spleen cells all contained very low titers of costimulator, as assayed on dilute, fresh thymocytes. Mixing splenic Mφ with Mφ-depleted thymocytes induced the production of high levels of costimulator. Similar results, not shown, were obtained with spleen cells as the source of both Mφ and Mφ-depleted cells. In that case, as expected, depletion of Mφ was less complete, and the synergy was less marked (4- to 5-fold) than in the case of depleted thymocytes. The relative amounts of costimulator produced by spleen and thymus cells is illustrated by the data of Tables II and III. As already indicated, normal spleen cells are a more productive source.

Costimulator requires a lectin to induce proliferation. Since Con A was essential to induce the release of costimulator, it was not possible to determine directly whether crude preparations of costimulator could induce mitogenesis in the absence of the lectin. Dilution experiments suggested that much lower levels of Con A could support costimulator-mediated mitogenesis than were normally used. It was possible, nevertheless, that either the lower levels of Con A were still required, or that an altered form of Con A, perhaps analogous to dimeric Con A (15), was responsible for costimulator activity. The mitogenic activity of crude costimulator preparations was blocked by the addition of 0.1 M MAG, a specific inhibitor of Con A-mediated mitogenesis (16, 17). Thus, either costimulator is an altered form of Con A which effects mitogenesis in an MAG-sensitive way, or there is a separate requirement for a lectin, as well as for costimulator. The second mechanism is shown to be correct by the following experiments, described in Table IV. First, PHA was found to be very poorly mitogenic and MAG had no effect on this (lines 1 and 2). Costimulator, in the presence of 1 μg/ml Con A carried over from the initial culture, induced a moderate rate of proliferation, but this was almost completely blocked by 0.1 M MAG (lines 3 and 4). However, in the presence of costimulator and MAG, low levels of PHA now induced strong proliferative activity. Thus, PHA, which is poorly mitogenic alone, can nevertheless supply a necessary component of mitogenesis, this component being missing when the residual Con A in crude costimulator preparations is neutralized by MAG. Clearly, costimulator activity resides in a molecule other than Con A, and costimulator is not in itself mitogenic.

Effect of cell contact. To see if cell contact stimulated mitogenesis, cultures were set up in flat-bottomed glass vials, at a constant cell to volume ratio and varying cell number (and volume). The results (Fig. 3) indicate that at an optimal cell to surface area ratio, stimulation is earlier than at lower ratios. As discussed below, these data can be interpreted as demonstrating either the importance of cell-cell contact for stimulation, or

TABLE III

Synergy of thymic lymphocytes and splenic macrophages in costimulator production

Source of Costimulator	Activity
1. None (background)	0.8
2. Normal thymocytes	1.3
3. Mφ-depleted thymocytes	0.6
4. Splenic Mφ (irradiated)	2.2
5. Mφ-depleted thymocytes + splenic Mφ	8.1
6. 5/(4 + 3) (− background)	6.1

Various costimulator-containing media were generated by culturing the cells indicated for 18 hr, diluted 1:6 with normal medium, and assayed on fresh, dilute thymocytes (0.5×10^6/ml). Con A levels were as in Table II. Normal thymocytes (line 2) were cultured at 12×10^6/ml, as were Mφ-depleted thymocytes. Mφ depletion was by the carbonyl iron technique (12). Splenic macrophages were prepared by irradiation, followed by overnight culturing. The cells surviving from 12×10^6 starting spleen cells were added to each milliliter of the generating cultures in lines 4 and 5. Thymocyte assay cultures were assayed at 101 hr, and the DNA synthetic activity was measured as in Table I. Line 6 expresses the stimulation observed by mixing Mφ with lymphocytes (line 5) relative to the separate cell types (lines 3 and 4).

TABLE IV

Separate requirements for lectin and costimulator

Additions to Thymocyte Culture	Activity	
	72 hr	91 hr
(1) PHA, 1:200–1:25	1.1–2.6	1.7–2.2
(2) PHA, 1:200–1:25; MAG	0.8–1.7	0.8–1.6
(3) Costimulator, 1:6	18.9	42.6
(4) Costimulator, 1:6; MAG	3.9	5.2
(5) Costimulator, 1:6; MAG; PHA, 1:200	89	136

Fresh thymocytes were cultured at 0.5×10^6 cells/ml, and assayed for thymidine uptake rate at 72 and 91 hr. When present, MAG was at 0.1 M. Costimulator was produced in cultures of 16×10^6 spleen cells/ml incubated for 17 hr with 6 μg/ml Con A, and contributed 1 μg/ml Con A to the experimental cultures as used (diluted 1:6). Used by itself, PHA was poorly mitogenic over the dilution range given, the optimal value being 1:50–1:25. Used with costimulator, the optimal PHA dilution was 1:200.

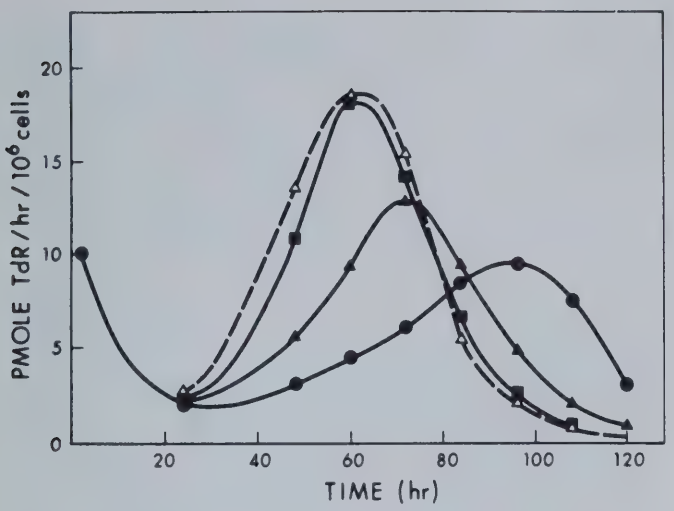

Figure 3. Effect of cell-to-surface area ratio on stimulation. Thymocytes were cultured at 2×10^6/ml in 7.5 μg/ml Con A in flat bottomed vials. The added cells produced the following densities; ●, $0.5 \times 10^6/cm^2$; ▲, $1 \times 10^6/cm^2$; ■, $2.5 \times 10^6/cm^2$; △, $5 \times 10^6/cm^2$.

the limited diffusion of a soluble costimulator. At a cell density of $2.5 \times 10^6/cm^2$, the cells, of diameter 6.3 μ, could form an average layer exactly one cell thick.

DISCUSSION

The most important result is the demonstration that primary thymocyte cultures are stimulated to synthesize DNA only if sufficient costimulator is present. Con A in itself is unable to induce mitogenesis at low cell concentrations. Since the cultures in Figure 1 contained identical numbers of cells, which could be seen to form comparable pellets on the rounded bottoms of the culture tubes, cell losses due to low numbers were obviated, and the lack thymidine uptake at low cell to volume ratios reflects lack of stimulation by Con A. The experiment of Figure 2 shows this more directly, in that medium from high density cultures will provide the costimulator. In principle, the conditioning effects of high density cultures could be due to removal of an inhibitory component of normal medium, or to secretion of a costimulator for mitogenesis. The stimulatory effect is transient at high cell densities (see Fig. 2). If the first mechanism were operative, it is unlikely that the removed inhibitor would reappear. Further, the costimulator activity is temperature sensitive (work in progress), arguing for a positive effector, rather than removal of a negative one. Finally, the high dilution at which costimulator preparations from cultured spleen cells are active (as high as 40-fold dilution with normal medium) indicates that a positive effector is present.

The costimulator activity generated at high cell densities and added to low density cultures allows the latter to continue DNA synthesis for a longer time than would the culture from which the costimulator was derived (Figs. 1, 2). The results may show that costimulator is used up at a rate dependent on the number of cells responding.

Several other costimulators, or potentiators, have been described. Human peripheral blood leukocytes potentiate their own response to PPD (18). A stimulating factor, specific for autologous lymphocytes, enhances mitogen or mixed lymphocyte responses (1). The effect of low cell density, delaying the PHA response of peripheral leukocytes (19), may have a similar origin. The level of thymic costimulator observed in our work is at least partly related to the time course of proliferation. That is, it is present at a time when cells are being recruited into the proliferative response, and declines before maximal stimulation occurs (Fig. 2). This supports the hypothesis that costimulator is essential for mitogenesis. Additional suggestive evidence for this is that spleen cultures, which yield higher titers of costimulator than do thymus cultures, are affected only marginally by dilution of cells (data not shown). However, although costimulator can be demonstrated to be essential for mitogenesis of thymus cell cultures, it is in itself insufficient; a lectin is also required. This requirement can be met by PHA, which by itself is a poor mitogen for these cells. Thus, there is a synergistic effect between added costimulator and PHA under conditions where residual Con A in the costimulator preparations is neutralized by methyl-α-glucoside (16, 17).

The cellular source of costimulator cannot yet be identified. Both macrophages and T lymphocytes (θ positive) are required for optimal production (Tables II and III). Data of other laboratories have been interpreted to show that either Mφ (2, 6, 7) or lymphocytes (5), produce lymphokines similar to costimulator (see *Introduction*). We urge caution in making firm conclusions for two reasons. First, it is difficult to prove that any cell population is "pure". Second, it is important to attempt quantitative assessments on the levels of lymphokines produced under various conditions. Thus, in Tables II and III, it is clear that significant levels of costimulator are produced by irradiated splenic Mφ, but it is also apparent that much higher levels are produced by the combinition of Mφ with θ-bearing lymphocytes.

Two properties of our costimulator differentiate it from most other reports. It is not a mitogen on its own, and it can easily be produced in large amounts. Its lack of mitogenic activity has been confirmed by partly purifying high-titer preparations of costimulator. Partially purified costimulator from which Con A has been removed by chromatography over Sephadex G-25 has no mitogenic activity, yet acts as a costimulator for the Con A-induced mitogenesis of thymocytes (work in progress).

The apparently stimulatory effects of optimal cell/bottom surface area (Fig. 3) may be due to a cell-cell physical interaction. On the other hand, limited diffusion of costimulator might produce the same results (20).

There is an intriguing parallel between the costimulator results and the mechanism of antigenic stimulation of lymphocytes. T cells appear to stimulate antigen-triggered B cells by specific and also nonspecific humoral factors. Also, the T cell mitogen Con A becomes mitogenic toward B cells in the presence of soluble factors produced by Con A-stimulated T cells (9). Our data indicate that the thymocyte subpopulation responsive to Con A requires a cell-produced costimulator to complete the stimulation signal.

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THE ROLE OF MACROPHAGES IN THYMOCYTE MITOGENESIS[1]

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The thymic lymphocytes of CBA/J mice respond to the mitogen Concanavalin A (Con A) only in the presence of adherent cells of the monocyte-macrophage series. Depletion of adherent cells abrogates the response, and macrophage-rich populations of cells restore it. The need for macrophages and mitogen is completely provided by irradiated splenic macrophages which have been exposed to Con A and washed free of the soluble mitogen. The mitogen-macrophage effect in this case is apparently not due to soluble factors.

Even more striking than the effect of macrophages on fresh cultures of thymic lymphocytes is their ability to restimulate quiescent cells 72 hr after their first stimulation with Con A. The quiescent cells respond immediately and quantitatively to Con A in the presence of fresh macrophages. This stimulation, like that of fresh thymocytes, is also controlled by a lymphokine ("costimulator") produced by mixing macrophages, mitogen, and T lymphocytes.

Our data suggest a model in which two signals are required for mitogenesis. First, the interaction of macrophage, T cell, and mitogen elicits a soluble costimulator, which is itself not mitogenic. Secondly, in the presence of costimulator, the mitogen (either soluble, or, more efficiently, bound to macrophages) induces a proliferative response in the T cell.

Macrophages (Mϕ)[4] play an essential role in the induction of both humoral (1, 2) and cell-mediated (3) immunity. It has been less obvious that they are also required for mitogen-induced proliferative responses of lymphocytes. However, several studies support such a requirement; human peripheral lymphocytes (4) and guinea pig lymph node lymphocytes (5) do not respond to mitogens if they are depleted of adherent cells, and the response is restored by adding back Mϕ-enriched cell populations. These results are somewhat at odds with other data suggesting little dependence on Mϕ (6).

We have found that stimulation of mouse thymocytes is dependent on both a cell-produced costimulatory factor and a lectin (7). The costimulator is produced by the combination of Con A, T cells, and Mϕ. The Mϕ, but not the lymphocytes, can be heavily irradiated without affecting production of the costimulator. From these results one should expect to find that the Mϕ is essential to Con-A-induced mitogenesis of thymic lymphocytes. The present work will show this to be the case.

It is commonly observed that mitogen-stimulated lymphocytes undergo a limited phase of proliferation, and then become quiescent. During the quiescence of mouse thymocytes initially stimulated with Con A, we observed that the quiescence was not reversed by replacing the culture medium with fresh medium, nor was it due to cell death, the quiescent cells remaining viable for some time after DNA synthesis declined. In this paper, we describe how the quiescent cells can be stimulated to renewed activity, immediately and quantitatively, by added Mϕ. Both the initial requirement for Mϕ, and their ability to restimulate quiescent thymocytes, are at least partly replaceable by the factor we have called costimulator.

The role of Mϕ in the immune response has been proposed to include antigen "presentation" (8) and "processing" (1). A few studies have demonstrated that mitogen bound to, or carried internally by, Mϕ is an efficient stimulus to lymphocytes (9, 5). The present studies show this to be true for mouse thymic lymphocytes as well.

METHODS

Purification of viable lymphocytes. This was achieved by layering cells over Ficoll-metrizoate ('Lymphoprep', Nyegaard and Co, Oslo, Norway) according to the method of Böyum (10). This technique removes nonviable cells, which sediment to the bottom.

Macrophage depletion. This was achieved by incubating cells (*ca.* 3×10^7) with carbonyl iron (0.6 g), and removing adherent cells with a strong magnet (2). The yield of viable cells was approximately 50%.

Costimulator for mitogenesis (7) was obtained by incubating 16×10^6 spleen cells/ml for 24 hr with 1 μg/ml Con A in medium lacking fetal calf serum. The cell-free supernatant was concentrated 8-fold by freeze-drying, dialyzed against 0.01 M Tris (Cl^-) – 0.02 M NaCl, pH 8.0, and sterilized by filtration.

Other materials and methods were as described earlier (7, 11).

RESULTS

Macrophage dependence of the initial Con A response. Con A-stimulated CBA/J mouse thymocytes undergo a phase of limited DNA synthesis (7). After thymocytes were treated with carbonyl iron to remove adherent cells, they no longer gave a significant response ($\leq 7\%$) to Con A (Fig. 1). When 5×10^4 irradiated, Mϕ-enriched spleen cells were added to 2×10^6 Mϕ-depleted thymocytes, the DNA synthesis rate was 8 times as high as that of untreated thymocytes. Mϕ-depleted spleen cells did not reconstitute the depleted thymocytes. Thus, adherent cells of the monocyte-macrophage type are necessary for mitogenesis induced by Con A. For the purposes of this

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[2] G. M. received a Vessie Heckbert Memorial Fellowship.
[3] Please address correspondence to Verner Paetkau.
[4] Abbreviations used in this paper: Mϕ, macrophage(s); TdR, deoxythymidine; θ, theta antigen, found on T lymphocytes: MAG, α-methyl-glucopyranoside; POL, polymerized flagellar protein.

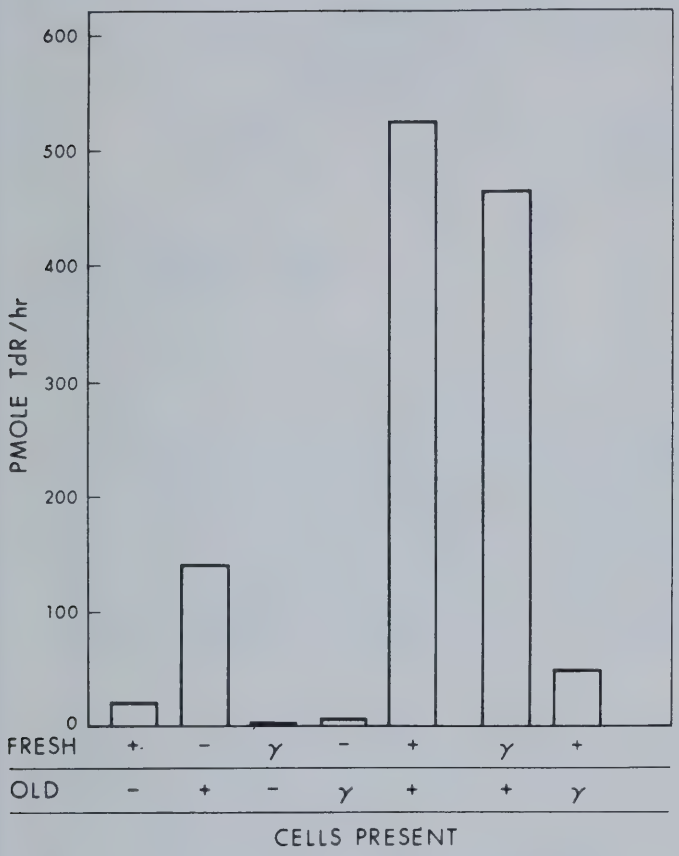

Figure 4. Determination of responding and stimulating cells. Combinations of harvested, Con A-stimulated, 72-hr-old cells and fresh thymus cells were mixed as indicated. Cells marked with γ were irradiated with a ^{137}Cs source (2500 rad) before mixing. The types of cells indicated on the abscissa were added to tubes at 1.7×10^6/ml. Cells were cultured in 2 ml of Con A medium, and harvested at 16 hr. The rate of DNA synthesis is expressed as pmole ^3H-TdR incorporated/hr/culture.

and these produced only a 1.4-fold increase in DNA synthesis of 72-hr thymocytes. Depletion of adherent cells from the spleen led to loss of stimulation by the surviving cells (data not shown).

Taken together, the properties of stimulating spleen cells (adherence, enhancement by 2-mercaptoethanol, radiation resistance) indicate that they are probably Mϕ.

Effect of cell to volume ratio on the stimulation of quiescent thymocytes by fresh cells. A soluble factor, called costimulator, is necessary for the initial Con A-induced response of thymocytes (7). Therefore, we determined if soluble, diffusible factors modified the restimulation of quiescent cells. When fixed numbers of quiescent (5×10^5) and fresh, irradiated (10×10^6) thymocytes were cultured in varying volumes of medium, the TdR uptake was maximized at 5×10^6 fresh cells/ml (Fig. 6). With higher cell concentrations, DNA synthesis may have been limited by medium depletion. The effect of diluting cells in this experiment, namely, a lower response, was probably due to dilution of costimulator, as further discussed below.

Stimulation of quiescent thymocytes by costimulator and mitogen. The stimulating effect of fresh, irradiated (Mϕ) cells on quiescent thymocytes depended on the mitogen, Con A, being present (data not shown). Costimulator preparations containing negligible levels of Con A (*Methods*) were not directly stimulatory toward quiescent thymocytes. Thus, 72-hr, quiescent cells, exposed to costimulator for the following 72 hr, gave a response of 5.5 pmole/hr/10^6 cells. When Con A was also present, the response was 46. Con A alone led to a rate of only 2.5. This is similar to the lack of direct mitogenic activity of costimulator toward fresh thymocytes (7). However, with Con A present, costimulator elicited both a DNA synthe-

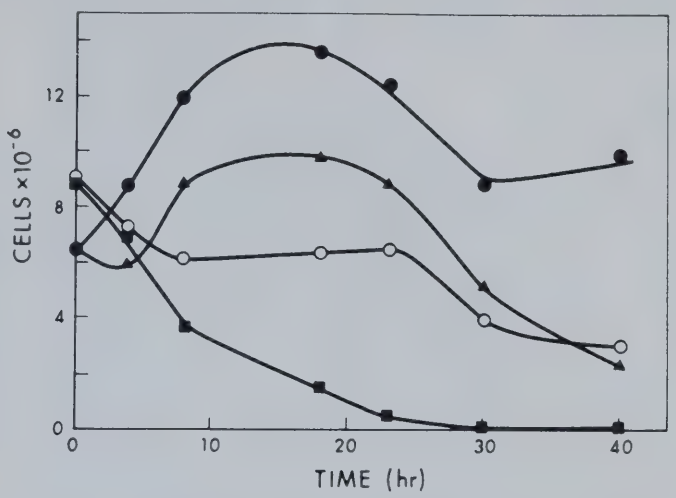

Figure 5. Cell proliferation after mixing quiescent thymocytes with fresh, irradiated thymocytes. Cells cultured in Con A for 72 hr were collected and treated as described (*Methods*) to remove non-viable cells. Viable old cells (6×10^6) were then cultured with 9×10^6 irradiated macrophage-containing thymocytes in 4 ml of medium with Con A. The numbers of viable cells (eosin exclusion), and the total cell numbers, were determined at the times indicated. At least 100 viable cells were counted (except in the case of irradiated fresh cells alone). The corrected total (●) and viable (▲) cell counts were obtained by taking the counts from old cell-irradiated fresh cell mixtures and subtracting the total (○) and viable (■) cells seen in a tube that contained only irradiated cells.

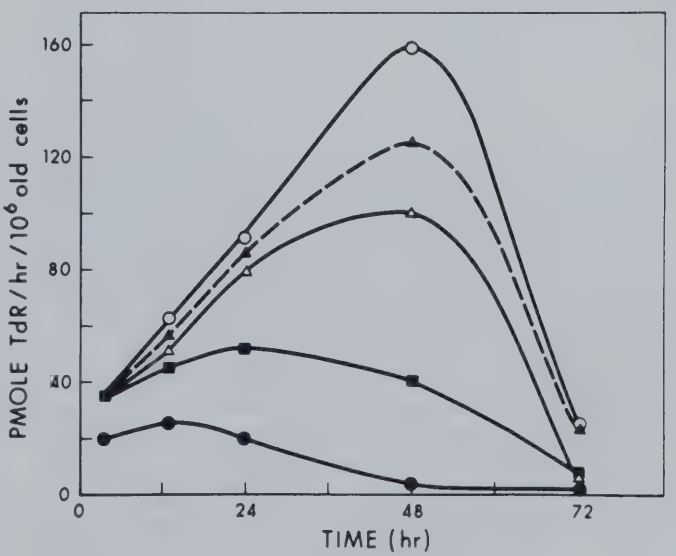

Figure 6. Effect of decreasing cell to volume ratio. Old (quiescent) cells (72 hr) were pooled and collected. One-half million of these cells were cultured in 2 ml medium (●), or with 10×10^6 irradiated, macrophage-containing thymocytes, in 1 ml (▲), 2 ml (○), 4 ml (△), or 8 ml (■) of fresh Con A medium. The cells were harvested at the times indicated on the abscissa. The late time of maximal DNA synthesis (48 hr) is due to the smallness of the number of responding (old) cells, and to the effectiveness of stimulation by the large number of fresh, irradiated cells.

TABLE IV

Response of 72-hr (quiescent) thymocytes to costimulator and macrophages[a]

Stimulant Added to 72-hr Cells	Relative Cell No.		DNA Synthesis rate (pmole/hr/10^6 cells), 48 hr
	48 hr	72 hr	
1. Con A	0.25	0.08	2.2
2. 1/50 costimulator + Con A	4.4	6.3	89
3. 1/100 costimulator + Con A	2.7	4.1	45
4. 1/200 costimulator + Con A	3.0	1.6	17.8
5. Mϕ + Con A	5.6	15.5	65
6. Con A-bearing Mϕ (no free Con A)	8.6	5.4	18

[a] In each case, 5×10^5 viable, 72-hr thymocytes were cultured in 1 ml. The Mϕ (line 5) were the surviving cells from 2×10^6 fresh, irradiated spleen cells. Con A-bearing Mϕ were prepared by incubating spleen cells for 4 hr at 37°C with 6 μg/ml Con A, washing two times, and irradiating them. The Mϕ contributed 1.7 pmole and 0.4×10^6 cells, at 48 hr, and 0.1×10^6 cells at 72 hr (determined by culturing them separately). The given cell numbers are corrected for this contribution, and expressed relative to the starting number of thymocytes. Con A was present at 3 μg/ml where indicated. The source of costimulator is described in the *Methods*. Under these conditions, it was not mitogenic by itself (see text).

sis and cell proliferation response (Table IV). Under the conditions of this experiment (5×10^5 thymocytes/ml) the restimulation was more drawn out than in the experiment of Figure 3 (2×10^6 thymocytes/ml) (see *Discussion*). At a dilution of 1:50, added costimulator induced a response comparable to that of splenic Mϕ. However, added Mϕ sustained the cell proliferation response for longer times (a 15.5-fold increase in cell number by 72 hr), perhaps by maintaining the viability of cells. In short, quiescent thymocytes are restimulated either by macrophages plus mitogen, macrophages carrying mitogen, or costimulator plus mitogen. Mitogen is essential; with costimulator provided, the macrophage may not be.

DISCUSSION

The data presented here add to the list of lymphocyte subpopulations requiring Mϕ for mitogenesis. Human peripheral leukocytes (4) and guinea pig lymph node cells (5, 12a) require Mϕ. In other systems this requirement is either less obvious (13), or not apparent at all (6). Possible reasons for this variability include the difficulty of removing all Mϕ or their monocyte precursors, and the variability in Mϕ requirements of different lymphocyte subpopulations, or mitogens, or both. In this regard, the T cell-independent antigen, polymerized flagellar protein (POL) of *Salmonella adelaide* appears to have a lower, yet significant, Mϕ requirement than does the T cell-dependent anti-sheep erythrocyte response (2). In any case, a positive result demonstrating Mϕ dependence is more convincing than negative results suggesting the opposite.

The role of Mϕ in mediating thymocyte mitogenesis is apparently 2-fold. First, Mϕ are required (as are θ-bearing lymphocytes) for the release of costimulator (7). Costimulator, in the presence of Con A, may replace Mϕ at the subsequent step. In the usual case, the Mϕ presents Con A to the thymic lymphocyte as the second step. Whether the apparent lack of Mϕ requirement in the presence of added costimulator (Table IV) is real or not, is again difficult to prove. However, it is apparent that Mϕ-presented Con A provides an efficient mitogenic stimulus, either for the initial response or for restimulation of quiescent thymocytes.

The ease of demonstrating a Mϕ requirement by quiescent cells lies partly in the depletion of Mϕ function in such cultures. We have observed (unpublished) that splenic Mϕ function is largely lost by 48 hr in Con A-containing cultures. The limiting number of thymic Mϕ are more easily depleted than are splenic Mϕ, and appear to limit both the initial thymocyte response (Fig. 1) and production of costimulator (7). In keeping with the above, thymocyte cultures become increasingly sensitive to stimulating effect of fresh Mϕ with time in Con A.

From the magnitude of response in the presence of added Mϕ, essentially all of the viable, quiescent thymocytes can be stimulated. The nature of the responding population has not yet been investigated, but it requires Con A stimulation for the first 72 hr to be generated (unpublished). The kinetic course of restimulation depends on the numbers of cells present. Specifically, small numbers of lymphocytes respond slowly, reaching a maximum 48 hr or later after restimulation. This may be due to a slow release and buildup of costimulator. The very rapid response of concentrated, 72-hr thymocytes to large numbers of fresh cells (Fig. 3) suggests that his system may be a useful model for the control of lymphocyte proliferation. The usual initial response of lymphocytes to mitogens leads to initiation of DNA synthesis at 24 hr, and recruitment of cells over a long time period (highly heterogeneous response) (11). Furthermore, only a fraction of the cells responds. Such responses make it difficult to relate early biochemical alterations, like cAMP (14) or membrane fluidity (15) changes, to the subsequent proliferation. The restimulated quiescent cells provide an alternative model in which all the cells respond, and respond immediately. Another useful aspect of this work is the ability to produce long term cultures of normal thymocytes with the possibility of inducing a variety of T cell functions (16). We have had cells proliferating for 200 hr and showing no signs of degeneration by adding costimulator or Mϕ periodically. Increases in cell number as high as 50-fold have now been achieved. It is rare to find net-fold increases in cell numbers after mitogen stimulation of normal lymphocytes. (A 3-fold increase in PHA-stimulated human peripheral lymphocytes in 5 days was observed by Jaehning *et al.* (17).)

The mechanism whereby Mϕ control thymocyte proliferation is not entirely clear. We have attempted to culture Mϕ and depleted thymocytes in divided cultures (sharing the same medium), so far without success (G.M., unpublished). This suggests that cell-cell contact is important, presumably for costimulator production, since once formed, costimulator can largely replace Mϕ. Nevertheless, a number of studies indicate that soluble, Mϕ-derived factors can replace Mϕ (5, 18, 19). These factors, however, appear to be produced in small amounts and are sometimes difficult to demonstrate except in double-chamber experiments (5). Furthermore, some appear to be mitogenic in themselves (19, 20); costimulator clearly is not (7; V.M. unpublished). Thus it would be premature to attempt comparisons in detail. Our analysis of the events occurring in stimulated thymocytes is clearly limited to this system. Since the Mϕ appears to have a rapid and early effect, as it is thought also to have in antigen responses (21), it is tempting to speculate that the primary effect of Mϕ is in fact to control the initiation of proliferative responses by lymphocytes. The role of costimulator in this process may make it similar to the T cell-elaborated factor which enables B cells to respond to Con A (22).

The model suggested by our data is that of two-step activa-